ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TERIFLUNOMIDE TABLETS DOSAGE FORM BY RP-HPLC

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032.

In partial fulfillment of the requirements for the award of Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted By

S.DEEPA

(Reg. No: 261430962)

Under the guidance of

Prof., Dr.D.Babu Ananth, M.Pharm.,Ph.D.,

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

APRIL 2016

Prof., Dr.D.Babu Ananth, M.Pharm.,Ph.D.,

Principal cum Professor,

Department of Pharmaceutical Analysis,

Edayathangudy.G.S.Pillay College of Pharmacy,

Nagapattinam – 611 002.

CERTIFICATE

This is to certify that the dissertation entitled **"ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TERIFLUNOMIDE TABLETS DOSAGE FORM BY RP-HPLC"** submitted by **S.Deepa** (Reg. No: 261430962) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in the Department of Pharmaceutical analysis, Edayathangudy.G.S Pillay College of Pharmacy during the academic year 2014-2016.

Place: Nagapattinam **(Prof., Dr.D.Babu Ananth, M.Pharm.,Ph.D.,)**

Date:

Prof., Dr.D.Babu Ananth, M.Pharm.,Ph.D.,

Principal cum Professor,

Department of Pharmaceutical Analysis,

Edayathangudy.G.S.Pillay College of Pharmacy,

Nagapattinam – 611 002.

CERTIFICATE

This is to certify that the dissertation **"ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TERIFLUNOMIDE TABLETS DOSAGE FORM BY RP-HPLC"** submitted by **S.Deepa** (Reg. No: 261430962) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under the guidance of **Prof., Dr.D.Babu Ananth, M.Pharm.,Ph.D.,** Director cum Professor, Edayathangudy.G.S Pillay College of Pharmacy during the academic year 2014 - 2016.

Place: Nagapattinam (**Prof.Dr.D.Babu Ananth, M.Pharm., Ph.D.,**)

Date:

ACKNOWLEDGEMENT

I would like to express profound gratitude to **Mrs.Jothimani G.S.Pillay,** Chairman, E.G.S.Pillay College of Pharmacy, and **Thiru. S.Paramesvaran, M.Com., FCCA.,** Secretary, E.G.S.Pillay College of Pharmacy.

I express my sincere and deep sense of gratitude to my Guide **Prof.Dr.D.Babu Ananth, M.Pharm., Ph.D.,** Department of Pharmaceutical Analysis E.G.S.Pillay College of Pharmacy, for his invaluable and extreme support, encouragement, and co-operation throughout the course of my work.

It is my privilege to express my heartfelt thanks to **Prof. Dr.D.Babu Ananth, M.Pharm, Ph.D.,** Principal, E.G.S.Pillay College of Pharmacy, for providing me all facilities and encouragement throughout the research work.

I express my sincere gratitude to **Prof. Dr.M.Murugan, M.Pharm., Ph.D.**, Director cum Professor, Head, Department of Pharmaceutics. E.G.S.Pillay College of Pharmacy, for his encouragement throughout the course of my work.

I express my sincere gratitude to **Prof., K.Shahul Hameed Maraicar, M.Pharm., (Ph.D).,** Director cum Professor, Department of Pharmaceutics. E.G.S.Pillay College of Pharmacy, for his encouragement throughout the course of my work.

I would like to extend my thanks to all the **Teaching Staff** and **Non Teaching Staff**, who are all supported me for the successful completion of my project work.

Last but not least, I express my deep sense of gratitude to my parents, family members and friends for their constant valuable blessings and kindness.

INDEX

LIST OF ABBREVIATIONS

API	: Active Pharmaceutical Ingredient
°C	: Degree centigrade
CSP	: Chiral Stationary Phase
FDS	: Forced Degradation Studies
Fig.	: Figure
ELSD	: Evaporative Light Scattering Detector
GHP	: G Hydrophilic Polypropylene
HETP	: Height Equivalent to a Theoretical Plate
HPLC	: High Performance Liquid Chromatography
HPTLC	: High Performance Thin Layer Chromatography
IS	: Internal Standard
LC	: Liquid Chromatography
Mg	: Milligram
ml	: Milli litre
nm	: Nanometer
NMR	: Nuclear Magnetic Resonance
NMT	: Not More Than
NP	: Normal Phase

ODS : Octa Decyl Silane

PDA : Photo Diode Array

PMR : Proton Magnetic Resonance

PVDF : Polyvinylidene difluoride

RI : Refractive Index

RSD : Relative Standard Deviation

RS : Reference Standard

SD : Standard Deviation

TF : Tailing Factor

USP : United States Pharmacopoeia

VWD : Variable Wavelength Detector

WRS : Working Reference Standard

µg /ml : Microgram per milli liter

µg : Microgram

1.0 INTRODUCTION

1.1 High-Performance Liquid Chromatography (HPLC)

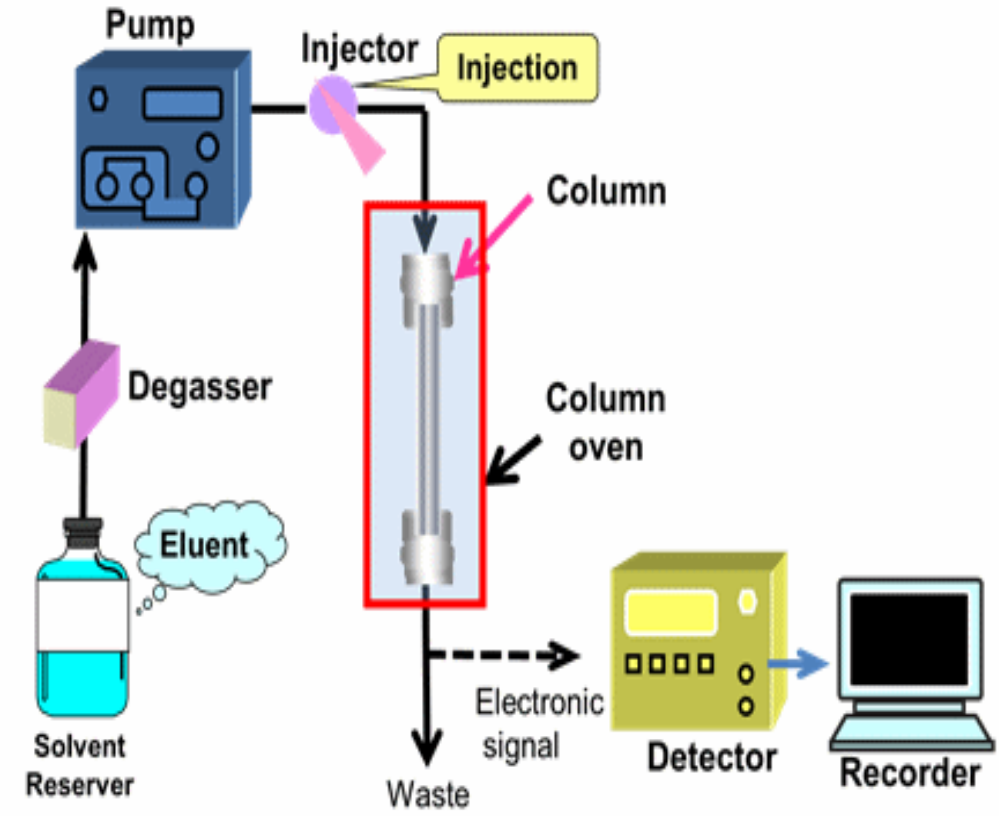
High-performance liquid chromatography, is a chromatographic technique used to separate the components in a mixture, to identify each component, and to quantify each component. The method involves a liquid sample being passed over a solid adsorbent material packed into a column using a flow of liquid solvent. Each analyte in the sample interacts slightly differently with the adsorbent material, thus retarding the flow of the analytes. If the interaction is weak, the analytes flow off the column in a short amount of time, and if the interaction is strong, then the elution time is long. **(Sharma B.K, 1994).**

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. **(Chatwal G.R, 1998)**

The HPLC method was considered the choice of estimation, since this method is the most powerful of all chromatographic and other separative methods. The HPLC method has enabled analytical chemist to attain great success in solving his analytical problems. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise, and accurate and the limit of detection is low and also it offers the following advantages. **(Beckett, 2002)**

The schematic representation of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates

a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or Refractive Index (RI).



SCHEMATIC REPRESENTATION OF HPLC SYSTEM

1.2 Components Of HPLC System

1.2.1 Pump

Pump generates a flow of eluent from the solvent reservoir to the system. Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses". There have been large system improvements to reduce this pulsation and the recent pumps create much less pulse compared to the older ones. Recent analysis requires very high sensitivity to quantify a small amount of analytes, and thus even a minor change in the flow rate can influence the analysis. Therefore, the pumps required for the high sensitivity analysis needs to be highly precise.

1.2.2 Injector

An injector is placed next to the pump. The simplest method is to use a syringe, and the sample is introduced to the flow of eluent. Since the precision of LC measurement is largely affected by the reproducibility of sample injection, the design of injector is an important factor. The most widely used injection method is based on sampling loops. The use of autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

1.2.3 Column

The separation is performed inside the column; therefore, it can be said that the column is the heart of an LC system. The packing material generally used is silica or polymer gels. The eluent used for LC varies from acidic to basic solvents. Most column housing is made of stainless steel, since stainless is tolerant towards a large variety of solvents. However, for the analysis of some analytes such as biomolecules

and ionic compounds, contact with metal is not desired, thus polyether ether ketone (PEEK) column housing is used instead.

Table 1 - COLUMN SELECTION FLOW CHART

Sample	LC mode	Column choice
Basic or Acidic	Reverse Phase-ion pair (allows neutral and charged compounds to be simultaneously analyzed)	C_{18}, C_8, C_6, C_4, C_2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)
	Ion suppression	C_{18}, C_8, C_6, C_4, C_2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)
Ionizable	Ion Exchange	
	Anionic →	Strong Anion exchange
	Cationic →	Strong Cation exchange
Neutral	Normal phase	Increasing polarity of bonded phases diol CN NH_2 Silica Alumina
	Reverse phase	Increasing polarity of bonding phase C_{18} C_8 Phenyl C_2 TMS CN

1.2.4 Detector

Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation. The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences. This difference is monitored as a form of electronic signal. **(Snyder L.R, Kirkland J.J 1983)**

1.2.5 On-line detectors:

- Refractive index
- UV/Vis Fixed wavelength
- UV/Vis Variable wavelength
- UV/Vis Diode array
- Fluorescence
- Conductivity
- Mass-spectrometric (LC/MS)
- Evaporative light scattering

1.2.6 Off-line detector:

- FTIR spiral disk monitor; requires sample transfer on the germanium disk and the following scanning in FTIR instrument.

1.2.7 Recorder

The change in eluent detected by a detector is in the form of electronic signal, and thus it is still not visible to our eyes. Nowadays, computer based data processor (integrator) is more common. There are software that are specifically designed for LC system. It provides not only data acquisition, but features like peak-fitting, base line correction, automatic concentration calculation, molecular weight determination, etc.

1.2.8 Degasser

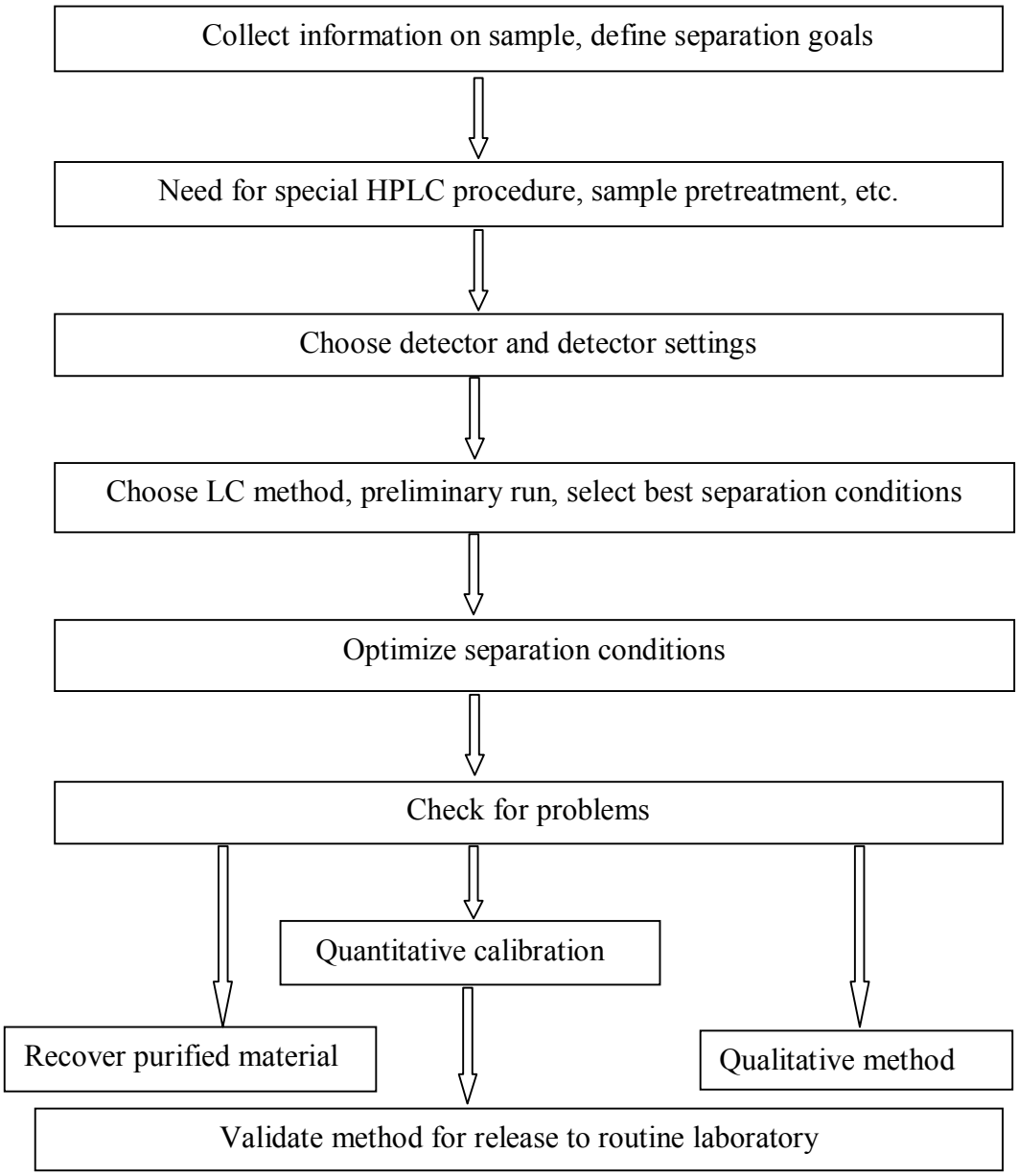
The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes. When gas is present in the eluent, this is detected as a noise and causes unstable baseline. Generally used method includes sparging (bubbling of inert gas), use of aspirator, distillation system, and/or heating and stirring. However, the method is not convenient and also when the solvent is left for a certain time period (e.g., during the long analysis), gas will dissolve back gradually. Degasser uses special polymer membrane tubing to remove gases. The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore. By placing this tubing under low pressure container, it created pressure differences inside and outside the tubing (higher inside the tubing). This difference let the dissolved gas to move through the pores and remove the gas. Compared to classical batch type degassing, the degasser can be used on-line, it is more convenient and efficient.

1.2.9 Column Heater

The LC separation is often largely influenced by the column temperature. Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperature (50~80°C). It is also important to keep stable temperature to obtain repeatable results even it is analyzed at around room temperature. There are possibilities that small different of temperature causes different separation results. Thus columns are generally kept inside the column oven (column heater).

1.3 Introduction To HPLC Method Development

Method development has following steps: **(Willard H.H, Merritt L.L, Dean J.A. and settle F.A,7th Edn)**



A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modelling. **(Synder K.L, Krikland J.J and Glajch J.L, 1983).**

1.4 Important factors to obtain reliable method

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are:

1. Careful sampling and sample preparation.
2. Appropriate choice of the column.
3. Choice of the operating conditions to obtain the adequate resolution of the mixture.
4. Reliable performance of the recording and data handling systems.
5. Suitable integration/peak height measurement technique.
6. The mode of calculation best suited for the purpose
7. Validation of the developed method.

1.4.1 Careful Sampling And Sample Preparation

Before beginning method development, it is need to review what is known about the sample in order to define the goals of separation. The sample related information that is important is summarized in following Table 2

Table: 2

Number of compounds present
Chemical structures
Molecular weights of compounds
pK_a values of compounds
UV spectra of compounds
Concentration range of compounds in samples of interest
Sample solubility

The chemical composition of the sample can provide valuable clues for the best choice of initial conditions for an HPLC separation.

1.4.2 Separation Goals (Snyder *et al*., 1997, Sharma B.K., 1980)

The goals of HPLC separation need to be specified clearly, which include:

- The use of HPLC to isolate purified sample components for spectral identification or quantitative analysis.
- It may be necessary to separate all degradants or impurities from a product for reliable content assay.
- In quantitative analysis, the required levels of accuracy and precision should be known (a precision of ± 1 to 2% is usually achievable).
- Whether a single HPLC procedure is sufficient for raw material or one or more different procedures are desired for formulations.
- When the number of samples for analysis at one time is greater than 10, a run time of less than 20 minutes often will be important.

1.4.3 Sample preparation

Samples come in various forms:

- Solutions ready for injection
- Solutions that require dilution, buffering, addition of an internal standard or other volumetric manipulation
- Solids must be dissolved or extracted
- Samples that require pretreatment to remove interferences and/or protect the column or equipment from damage.

Most samples for HPLC analysis require weighing and /or volumetric dilution before injection. Best results are often obtained when the composition of the sample solvent

is close to that of the mobile phase since this minimizes baseline upset and other problems. Some samples require a partial separation (pretreatment) prior to HPLC, because of need to remove interferences, concentrate sample analytes or eliminate "column killers".

The samples may be of two types, regular or special. The regular samples are typical mixtures of small molecules (<2000Da) that can be separated by normal starting conditions. Whereas special samples are better separated under customized conditions given in Table: 3

Table 3

Sample	Requirements
Inorganic ions	Detection is primary problem; use ion chromatography.
Isomers	Some isomers can be separated by reversed-phase HPLC and are then classified as regular samples; better separations of isomers are obtained using either (1) normal-phase HPLC or (2) reversed-phase separations with cyclodextrin-silica columns.
Enantiomers	These compounds require "chiral" conditions for their separation.
Biological	Several factors make samples of this kind "special": molecular conformation, polar functionality, and a wide range of hydrophobicity.
Macromolecules	"Big" molecules require column packings with large pores (>>10-nm diameters); in addition, biological molecules require special conditions as noted above.

1.4.4 Choice of the Column

The selection of the column in HPLC is somewhat similar to the selection of columns in G.C, in the sense that, in the adsorption and partition modes, the separation mechanism is based on inductive forces, dipole-dipole interactions and hydrogen bond formation. In case of ion-exchange chromatography, the separation is based on the differences in the charge, size of the ions generated by the sample molecules and the nature of ionisable group on the stationary phase. In the case of size-exclusion chromatography the selection of the column is based on the molecular weight and size of the sample components. Selection of columns based on the method is briefly summarized in Table 4.

Table: 4

Method/ Description/Columns	**Preferred Method**
Reversed-phase HPLC Uses water- organic mobile phase Columns: C_{18} (ODS), $C_{8,}$ phenyl, trimethylsilyl (TMS), cyano.	First choice for most samples, especially neutral or non-ionized compounds that dissolve in water-organic mixtures
Ion-pair HPLC Uses water-organic mobile phase, a buffer to control pH, and an Ion-pair reagent Columns: C_{18}, C_{8}, Cyano	Acceptable choice for ionic or ionisable compounds, especially bases or cations.

Method/ Description/Columns	Preferred Method
Normal-phase HPLC Uses mixtures of organic solvents as mobile phase Columns: cyano, diol, amino, silica	Good second choice when reversed-phase or ion-pair HPLC is ineffective; first choice for lipophilic samples that do not dissolve well in water-organic mixtures; first choice for mixtures of isomers and for preparative HPLC

1.4.5 Operating conditions to obtain the adequate resolution of the mixture

Most of the drugs come under the category of regular samples. Regular samples mean typical mixtures of small molecules (<2000Da) that can be separated using more or less standardized starting conditions. Regular samples can be further classified as neutral or ionic. Samples classified as ionic include acids, bases, amphoteric compounds and organic salts. If the sample is neutral, buffers or additives are generally not required in the mobile phase.

Acids or bases usually require the addition of a buffer to the mobile phase. For basic or cationic samples, less acidic reverse phase columns are recommended. Based on recommendations of the conditions, the first exploratory run is carried and then improved systematically. On the basis of the initial exploratory run isocratic or gradient elution can be selected as most suitable. If typical reverse-phase conditions provided inadequate sample retention, it suggests the use of either ion-pair or normal phase HPLC. Alternatively, the sample may be strongly retained with 100% ACN as

mobile phase suggesting the use of non-aqueous reverse phase chromatography or normal phase HPLC.

1.4.6 Method Development

One approach is to use an isocratic mobile phase of some average organic solvent strength (50%). A better alternative is to use a very strong mobile phase first (80-100%) then reduce %B as necessary. The initial separation with 100% B results in rapid elution of the entire sample, but few groups will separate. Decreasing the solvent strength shows the rapid separation of all components with a much longer run time, with a broadening of latter bands and reduced retention sensitivity. Goals that are to be achieved in method development are briefly summarized in Table 5.

Table: 5

Goal	**Comment**
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures.
Quantitation	≤2% for assays; ≤5% for less-demanding analyses ≤15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent consumption	Minimum mobile-phase use per run is desirable.

Separation or resolution is a primary requirement in quantitative HPLC. The resolution (Rs) value should be maximum (Rs > 1.5) favors maximum precision. Resolution usually degrades during the life of the column and can vary from day to day with minor fluctuations in separation conditions. Therefore, values of Rs=2 or greater should be the goal during method development for sample mixtures. Such resolution will favor both improved assay precision and greater method ruggedness.

Some HPLC assays do not require base line separation of the compounds of interest (qualitative analysis). In such cases only enough separation of individual components is required to provide characteristic retention times for peak identification.

The time required for a separation (runtime = retention time for base band) should be as short as possible and the total time spent on method development is reasonable (runtimes 5 to 10 minutes are desirable).

Conditions for the final HPLC method should be selected so that the operating pressure with a new column does not exceed 170 bar (2500 psi) and an upper pressure limit below 2000 psi is desirable. There are two reasons for this pressure limit, despite the fact that most HPLC equipment can be operated at much higher pressures. First, during the life of a column, the backpressure may rise by a factor of as much as 2 due to the gradual plugging of the column by particulate matter. Second, at lower pressures (<170 bars) pumps, sample valves and especially auto samplers operate much better, seals last longer, columns tend to plug less and system reliability is significantly improved. For these reasons, a target pressure of less than 50 % of the maximum capability of the pump is desirable. When dealing with more challenging

samples or if the goals of separation are particularly stringent, a large number of method development runs may be required to achieve acceptable separation.

1.4.7 Repeatable Separation

As the experimental runs described above are being carried out, it is important to confirm that each chromatogram can be repeated. When we change conditions (mobile phase, column, and temperature) between method development experiments, enough time must elapse for the column to come into equilibrium with the new mobile phase and temperature.

Usually column equilibration is achieved after passage of 10 to 20 volumes of the new mobile phase through the column. However, this should be confirmed by repeating the experiment under the same conditions. When constant retention times are observed in two such back-to-back repeat experiments (± 0.5% or better), it can be assumed that the column is equilibrated and the experiments are repeatable.

1.4.8 Optimization of HPLC Method

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts, asymmetry, capacity factor, elution time, detection limits, limit of quantitation and overall ability to quantify the specific analyte of interest.

Optimization of a method can follow either of two general approaches:

- ❖ Manual
- ❖ Computer driven

The manual approach involves varying one experimental variable at a time, while holding all other constant and recording changes in response. The variables might include flow rate, mobile or stationary phase composition, temperature, detection

wavelength and P^H. This approach to system is slow, time consuming and potentially expensive. However, it may provide a much better understanding of the principles and theory involved and of interactions of the variables.

In the second approach, computer driven automated method development, efficiency is optimized while experimental input is minimized. This approach reduce the time, energy and cost of all instrumental method development.

The various parameters that include to be optimized during method development are

A. Selection of mode of separation.

B. Selection of stationary phase.

C. Selection of mobile phase.

D. Selection of detector.

1.4.8.1 Selection Of Mode Of Separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation .A second factor is the nature of the matrix.

1.4.8.2 Selection of stationary phase

Selection of the column is the first and the most important step in method development. The appropriate choice of separation column indicates three different approaches.

- Selection of separation
- The particle size and nature of the column packing
- The physical parameters of the column i.e. the length and the diameter

Some of the important parameters considered while selecting chromatographic columns are

- Length and diameter of the column
- Packing material
- Shape of the particles
- Size of the particles
- % of Carbon loading
- Pore volume
- Surface area
- Reproducibility and reliability
- End capping

In this case, the column selected had a particle size of 5µm and an internal diameter of 4.6mm.The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilities a wide range of columns like dimethyl silane (C_2), butysilane (C_4), octysilane(C_8), octadecylsane (C_{18}),base deactivated silane (C_{18}), BDS phenyl, cyanoproply (CN), nitro, amino etc. Silica based columns with different cross linking's in the increasing order of Polarity are as follows:

<……. Non-polar …… moderately polar……. Polar. ……>

C_{18} < C_8 < C_6 < Phenyl < Amino < Cyano < Silica

C_{18} was chosen for this study since it is most retentive one. The sample manipulation becomes easier with this type of column. Generally longer columns

provide better separation due to higher theoretical plate numbers. Columns with 5µm particle size give the best compromise of efficiency.

Peak shape is equally important in method development. Columns that provide symmetrical peaks are always preferred while peaks with poor asymmetry can result in,

- Inaccurate plate number and resolution measurement
- Imprecise quantitation
- Degraded and undetected minor bands in the peaks tail
- Poor retention reproducibility

A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

A column which gives separation of all the impurities and degradants from each other and from analyte peak and which is rugged for variation in mobile phase shall be selected.

1.4.8.3 Selection Of Mobile Phase

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from each other and from analyte peak.

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute-stationary phase, solute-mobile phase, and mobile phase-stationary phase. For a given stationary phase, the nature and the composition of which has to be judiciously selected in order

to get appropriate and required solute retention. The mobile phase has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation). Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the pKa of the analytes. The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- ❖ Buffer
- ❖ pH of the buffer
- ❖ Mobile phase composition

1.4.8.4 Buffers if any and its strength:

Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most commonly employed buffers are

- Phosphate buffers prepared using salts like KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4.
- Phosphoric acid buffers prepared using H_3PO_4.
- Acetate buffers-Ammonium acetate, Sodium acetate etc.
- Acetic acid buffers prepared using CH_2COOH.

The retention also depends on the molar strengths of the buffer-Molar strength is increasingly proportional to retention times. The strength of the buffer can be increased, if necessary to achieve the required separations. The solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength. The useful pH range for

columns is 2 to 8, since siloxane linkages are cleaved below pH-2 while at pH values above eight, silica may dissolve.

1.4.8.5 Mobile phase composition:

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to the fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are Methanol and Acetonitrile. Experiments should be conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations of analyte peak. A mobile phase which gives separation of analyte peak and which is rugged for variation of both aqueous and organic phase by at least ± 0.2% of the selected mobile phase composition should be used.

1.4.8.6 Selection Of Detector

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, florescence, conductance, oxidation, reduction etc. The characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity facilitating trace analysis.
- Negligible baseline noise to facilitate lower detection.
- Large linear dynamic range.
- Low dead volume.
- Inexpensive to purchase and operate.

Pharmaceutical ingredients do not absorb all UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive

properties of the organic impurities and the active pharmaceutical ingredient is very helpful. For the greatest sensitivity λmax should be used. Ultra violet wavelengths below 200nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.

1.4.8.7 Performance Calculations

Carrying out system suitability experiment does the performance calculations. System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validations have been completed. The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation SD can be determined during validation.

System suitability might then require that retention times fall within a ±3 SD range during routine performance of the method.

The USP (2000) defines parameters that can be used to determine system suitability prior to analysis include plate number (n), tailing factor (T), resolution (R_S) and relative standard deviation (RSD) of peak height or peak area for respective injections.

The RSD of peak height or area of five injections of a standard solution is normally accepted as one of the standard criteria. For assay method of a major component, the RSD should typically be less than 1% for these five respective injections.

The plate number and/ or tailing factor are used if the run contains only one peak. For chromatographic separations with more than one peak, such as an internal standard assay or an impurity method expected to contain many peaks, some measure of separations such as R_S is recommended. Reproducibility of t_R or k value for a specific compound also defines system performance.

The column performance can be defined in terms of column plate number. As the plate count is more the column is more efficient.

1.4.9 METHOD VALIDATION

The word "Validation" means "Assessment" of validity or action of proving effectiveness.

1.4.9.1 Definition

ICH defines validation as "establish the documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes".

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated.

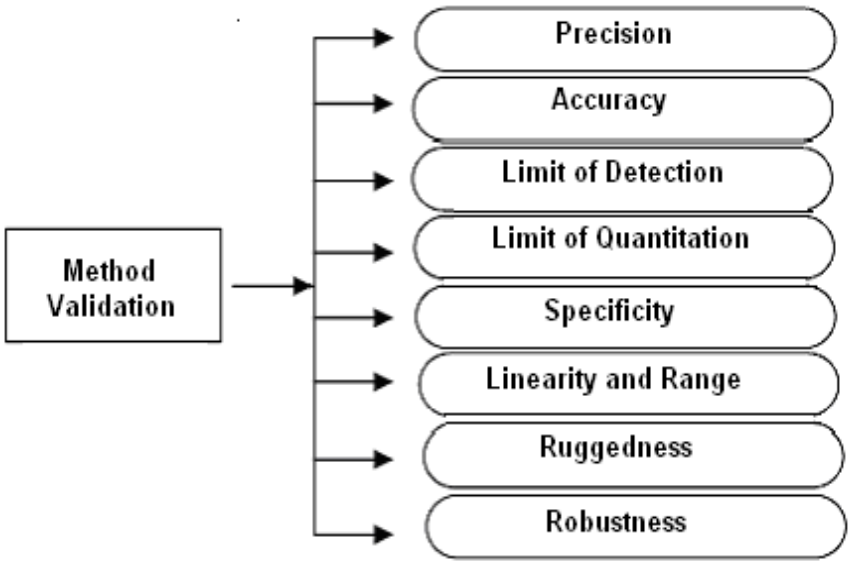
- Before their introduction into routine use
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics
- Whenever the method is changed, and the change is outside the original scope of the method.

1.4.9.2 Purpose Of Validation

- Enable the scientists to communicate scientifically and effectively on technical matter.
- Setting the standards of evaluation procedures for checking compliance and taking remedial action.
- Economic: Reduction in cost associated with process sampling and testing.
- As quality of the product cannot always be assured by routine quality control because of testing of statistically insignificant number of samples.
- Retrospective validation is useful for trend comparison of results compliance to CGMP/CGLP.
- Closure interaction with Pharmacopoeial forum to address analytical problems.
- International Pharmacopoeial harmonization particularly in respect of impurities determination and their limits.

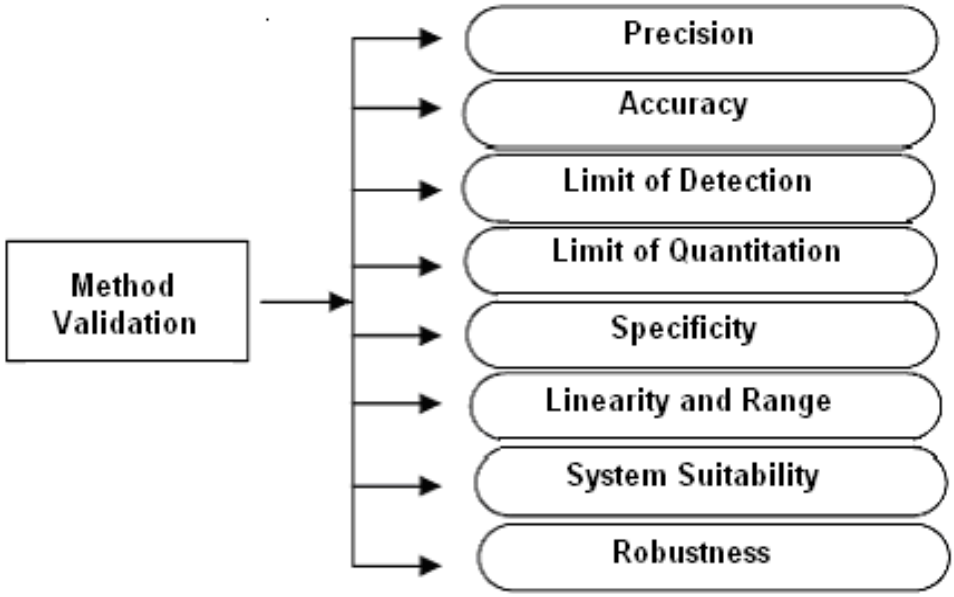
Method validation is completed to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use, and is sometime referred to as "the process of providing documented evidence that the method does what it is intended to do."

For method validation, these specifications are listed in USP Chapter <1225>, and can be referred to as the "Eight Steps of Method Validation," as shown in figure below. These terms are referred to as "**analytical performance parameters**", or sometimes as "analytical figures of merit."



The USP Eight Steps of Method Validation

In response to this situation, one of the first harmonization projects taken up by the ICH was the development of a guideline on the "Validation of Analytical Methods: Definitions and Terminology." ICH divided the "validation characteristics" somewhat differently, as outlined in Figure below



ICH Method Validation Parameters

1.4.9.3 Method Validation Parameters

The developed methods were validated by following steps:

A. Accuracy

It is defined as closeness of agreement between the actual (true) value and mean analytical value obtained by applying a test method number of times. Spike and recovery studies are performed to measure accuracy: a known sample is added to the excipients and the actual drug value is compared to the value found by the assay. Accuracy is expressed as the bias or the % error between the observed value and the true value (assay value/actual value x 100%).

The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method.

The parameter provides information about the recovery of the drug from sample and effect of matrix, as recoveries are likely to be excessive as well as deficient.

Procedure:

Use a minimum of 3 spiking concentrations in the excipient solution. Prepare two samples of each concentration. Test the 6 samples in triplicate on one run. Measure expected vs. average measured value. Calculate the % recovery.

B. Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample.

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation

(%RSD) or coefficient of variation (% CV) for a statistically significant number of samples. According to the ICH, precision should be performed at three different levels: *repeatability, intermediate precision, and reproducibility*.

Repeatability is the results of the method operating over a short time interval under the same conditions (or) is the % RSD of multiple determinations of a single sample in a single test run (intra-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration.

Procedure :

- Prepare three dilutions of the sample (high/medium/low concentrations in the range).
- Test 10 replicates of each dilution of the sample.
- Calculate the average and standard deviation for each point on the curve.
- Calculate the RSD for each point on the curve.

Intermediate precision is the results from within lab variations due to random events such as different days, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the individual variables can be monitored (or) intermediate precision (also called inter-assay precision) measures the % RSD for multiple determinations of a single sample, controls and reagents analyzed in several assay runs in the same laboratory.

Procedure:

- Prepare three dilutions of the sample (high/medium/low concentrations in the range).

- Test triplicates of each dilution of the sample in three different assays.
- Do for day-to-day variations
- Do for lot-to-lot variations of assay materials
- Do for technician-to-technician variation.
- Calculate the average and standard deviation for each point on the curve for each individual test.
- Calculate the RSD for each point on the curve between the assay runs.

Reproducibility refers to the precision between laboratories usually in collaborative studies and not directly relevant to assay validation in a manufacturing facility.

Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and the confidence interval.

C. Specificity

It is the ability of an analytical method to assess unequivocally the analyte of interest in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. It is not possible to demonstrate that an analytical procedure is specific for a particular analyte. In such case a combination of two or more analytical procedure is recommended to achieve the necessary level of discrimination. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures or tests.

In case of the assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substances or product with appropriate levels of impurities or excipients and demonstrating that the assay is unaffected by the presence of these extraneous materials. If the degradation product impurity standards are unavailable,

specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g., pharmacopoeia method or other validated analytical procedure (independent procedure). These comparisons should include samples stored under relevant stress conditions (e.g. light, heat humidity, acid/base hydrolysis, oxidation, etc.).

D. Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to-noise ratio, usually two- or three-to-one. The ICH has recognized the signal-to-noise ratio convention, but also lists two other options to determine LOD: visual non-instrumental methods and a means of calculating the LOD. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

Procedure

- Prepare a standard concentration of the product in the appropriate solution.
- Prepare a blank solution without any sample (zero concentration).
- Perform the assay at least 3 times in duplicate according to the SOP.
- Measure the amount present in the sample and blank.
- Calculate the average for the sample and blank.
- Calculate and standard deviation of the blank.
- Calculate the LOD as 3.3 x SD /slope of linearity curve.

E. Limit of Quantitation

The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. That is, as the LOQ concentration level decreases, the precision increases. If better precision is required, a higher concentration must be reported for LOQ.

Procedure:

The calculation method is again based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula: LOQ = 10(SD/S). Again, the standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines.

F. Linearity :

It is the ability of an assay to obtain test results, which are directly proportional to the concentration of an analyte in the sample. The determination of linearity will identify the range of the analytical assay. It can be measured as slope of the regression line and its variance or as the coefficient of determination (R^2) and correlation coefficient (R).

Procedure:

Determining the coefficient of correlation R for dilutions of the sample over the range claimed for the assay.

1. Prepare 6 to 8 sample dilutions across the claimed range
2. Test each dilution in triplicate for 3 runs
3. Record expected values, actual values, and % recoveries for each run

4. Analyze each set of dilutions as a linear curve and calculate R for each assay.

Alternative:

Calculate the accuracy and precision at each dilution. Range is the highest and lowest concentration with satisfactory accuracy and precision.

If the validation study for an analytical test is well planned it should be possible to design the protocol to consider many of the parameters in a single series of tests, for instance: selectivity (specificity) linearity, range, accuracy and precision for a potency test.

G. Range:

Range is the interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. If the relationship between response and concentration is not linear, the range may be estimated by means of a calibration curve.

The range is normally expressed in the same units as the test results obtained by the method. The ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges. For assay, the minimum specified range is from 80-120% of the target concentration. For an impurity test, the minimum range is from the reporting level of each impurity, to 120% of the specification. (For toxic or more potent impurities, the range should be commensurate with the controlled level).

H. Ruggedness:

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different

laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc.

I. Robustness:

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are:

- Stability of analytical solutions
- Extraction time

In the case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate.

In the case of gas-chromatography, examples of typical variations are

- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate.

J. System Suitability Test (SST)

SST is commonly used to verify resolution, column efficiency, and repeatability of the chromatographic system to ensure its adequacy for a particular analysis. According to the United States pharmacopoeia (USP) and the International Conference on Harmonization (ICH), SST is an integral part of many analytical procedure.

Primary SST parameters are most important as they indicate system specificity, precision and column stability. Other parameter include capacity factor (K) and signal to noise ratio (S/N) for impurity peaks.

The USP chromatography general chapter states **(USP 36–NF 31, 621 – Chromatography)**

"System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system can be evaluated as such."

1.5 Interpretation Of Chromatograms

Figure below represents a typical chromatographic separation of two substances, 1 and 2, where t_1 and t_2 are the respective retention times; and h, h/2, and $W_{h/2}$ are the height, the half-height, and the width at half-height, respectively, for peak 1. W_1 and W_2 are

the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid chromatography.

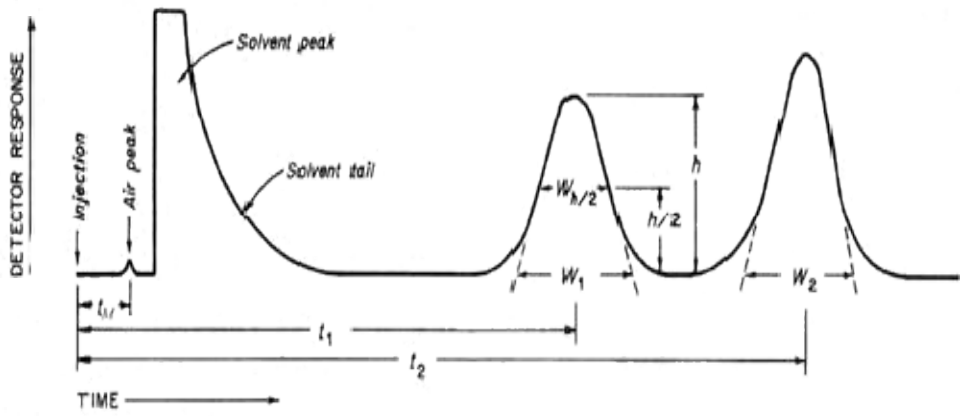


Figure - 1

Chromatographic retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next.

1.5.1 Relative Retention times:

Relative retention time is calculated by the equation $R_r = t_2/t_1$

t_2 = Retention time of test.

t_1 = Retention time of reference substance, determined under identical experimental conditions on the same column.

1.5.2 Relative Retention:

To calculate the relative retention (r) : $\dfrac{t_2 - t_M}{t_1 - t_M}$

where t_M is the retention time of the non-retained marker.

1.5.3 Resolution (as per USP) :

The resolution, R, is a function of column efficiency, N, and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug.

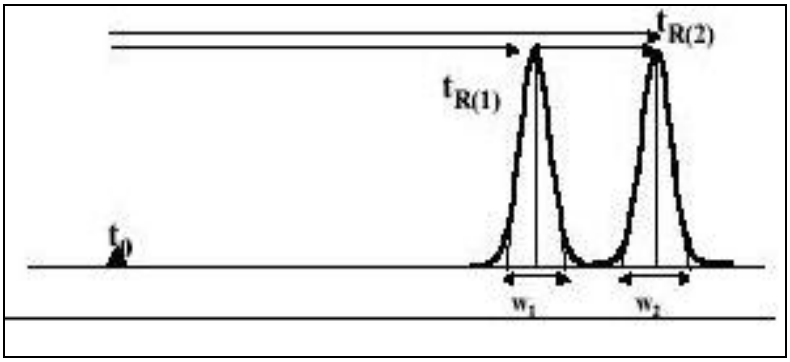


Figure - 2

R is determined by the equation:

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1}$$

Or

$$R = \frac{2(t_2 - t_1)}{1.70\ (W_{1,\,h/2} + W_{2,\,h/2})}$$

t_2 and t_1 are the retention times of the two components.

W_2 and W_1 are the corresponding width at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the base line.

$W_{1h/2}$ and $W_{2\,h/2}$ are the corresponding peak width at half-height.

1.5.4 Resolution (as per Ph.Eur) :

$$R = \frac{1.18(t_{R2} - t_{R1})}{(W_{h1} + W_{h2})}$$

Where, $t_{R2} > t_{R1}$

t_{R2} and t_{R1} = Retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks

W_{h1} and **W_{h2}** = peak width at half height

1.5.5 Theoretical Plates (as per USP and Ph.Eur)

Column efficiency also may be specified as system suitability requirements, especially if there is only one peak of interest in the chromatograms. The number of the theoretical plates, N, is a measure of column efficiency. It is calculated by the equation.

$$N = 16\left[\frac{t}{w}\right]^2 \text{ or } \qquad N = 5.54\left[\frac{t}{w_{1/2}}\right]^2$$

t = Retention time of the substance.

w = width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline.

w½ = Peak width at half-height.

1.5.6 Precision:

Precision a measure of either degree of reproducibility or of repeatability is determined by making replicate injections of standard preparation and calculating relative standard deviation. Unless otherwise specified in the individual monograph, data from five replicate injections of the standard preparation are used to calculate

the relative standard deviation (SR), if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

Relative Standard Deviation in percentage.

$$SR(\%) = \frac{100}{\bar{x}} \left[\frac{\sum_{i=1}^{N} (xi - \bar{x})^2}{N - 1} \right]^{1/2}$$

x = Arithmetic mean of the set.

xi = An individual measurement in a set of N measurements.

N = Number of individual values

1.5.7 Tailing Factor as per USP (or) Symmetry factor as per Ph.Eur:

Tailing factor, T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing factor is pronounced (Fig 1). In some cases values less than unity may be observed. As peak asymmetry increases, integration and hence precision becomes less reliable.

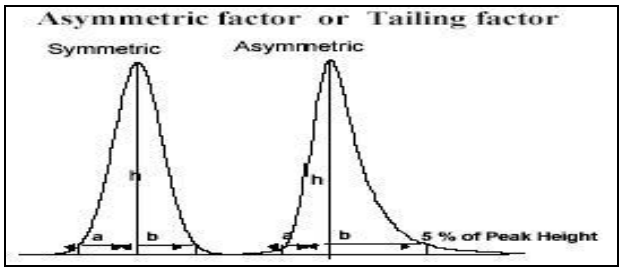


Figure - 3

$$\text{Tailing factor, } T = \frac{W0.05}{2f}$$

W0.05 = Width of peak at 5% height.

f = Distance from the peak maximum to the leading edge of the peak, the distance is being measured at a point 5% of the peak height from baseline.

1.5.8 Capacity Factor (Mass distribution ratio):

Capacity factor k' of a sample component is a measure of the degree which that component is retained by the column relative to an unretained component

Capacity factor is $k' = (t_r - t_0) / t_0$

t_r - is the elution time of retained component and

t_0 - is the elution time of the unretained sample.

1.5.9 Signal to Noise Ratio:

$$S/N = \frac{2H}{H}$$

Where,

H = Height of the peak corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height

h = Range of the background noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

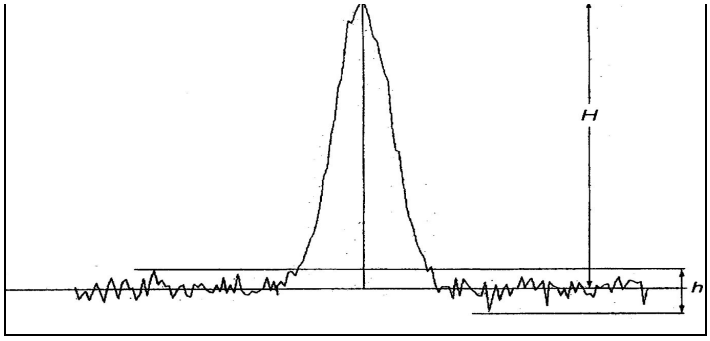


Figure - 4

1.5.10 Peak to valley ratio

The peak-to-valley ratio (p/v) may be employed as a system suitability requirement in a test for related substances when baseline separation between 2 peaks is not reached

$$p/v = \frac{H_p}{H_v}$$

H_p = Height above the extrapolated baseline of the minor peak,

H_v = Height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks.

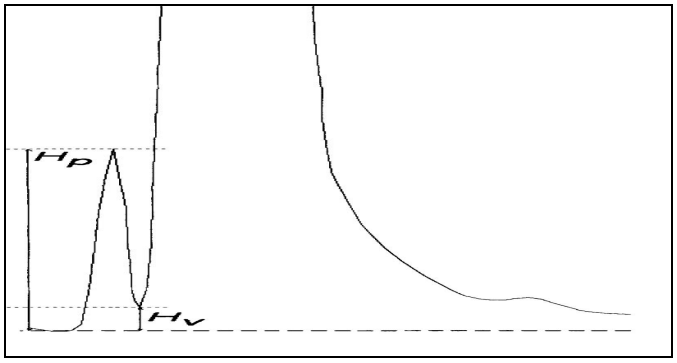


Figure - 5

System Suitability Parameters and Recommendations:

Parameter	**Recommendation**
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k'>2.0
Repeatability	RSD </= 1% for N >/= 5 is desirable
Relative retention	Not essential as long as the resolution is stated
Resolution	R_s of > 2 between the peak of interest and the closest eluting.
Tailing Factor (T)	T of </= 2
Theoretical Plates(N)	In general should be > 2000

1.6 Statistical Parameters

1.6.1 Linear regression:

Once a linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r', then the best straight line through the data points has to be estimated. This can often be done be done by visual inspection of the calibration graph, but in many cases it is far more sensible to evaluate the best straight line by linear regression (the method of least squares).

The equation of straight line is

y = mx + c

Where, y the dependent variable is plotted as result of changing x, the independent variable.

To obtain the regression line 'y on x' the slope 'm' of the line and the intercept 'c' on the y axis are given by the following equation.

$$\mathbf{m} = \frac{\mathbf{N\,\Sigma xy - (\Sigma x)\,(\Sigma y)}}{\mathbf{N\,\Sigma x2 - (\Sigma x)2}} \text{ and } \qquad \mathbf{c} = \frac{\mathbf{(\Sigma y)\,(\Sigma x2) - (\Sigma x)\,(\Sigma xy)}}{\mathbf{N\,\Sigma x2 - (\Sigma x)2}}$$

1.6.2 Correlation coefficient:

When the changes in one variable are associated or followed by changes in the other, it is called correlation. To establish whether there is a linear relationship between two variables x_1 and y_1, use Pearson's correlation coefficient r.

$$r = \frac{n\,\Sigma x1y1 - \Sigma x1y1}{\{[n\,\Sigma x12 - (\Sigma x1)2]\,[n\,\Sigma y12 - (\Sigma y1)2]\}1/2}$$

Where n is the number of data points.

The value of r must lie between +1 and -1, the nearer it is to +1, the greater the probability that a definite linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1 indicate negative

correlation values of 'r' that tend towards zero indicate that x and y are not linearly related (they made be related in a non-linear fashion).

1.6.3 Standard deviation:

The standard deviation measures the spread of the data about the mean value. It is commonly used in statistics as a measure of precision statistics as a measure of precision and is more meaningful than is the average deviation. It may be thought of as a root-mean-square deviation of values from their average and is expressed mathematically as

$$S = \sqrt{\frac{\sum_{i=1}^{i=n} (x_i - \bar{x})}{N - 1}}$$

Where,

S is standard deviation.

If N is large (50 or more) then of course it is immaterial whether the term in the denomination is N -1 or N

Σ = sum

x = observed values

$\bar{x}$ = Mean or arithmetic average= $\Sigma X/N$

$x - \bar{x}$ = deviation of a value from the mean

N = Number of observations

1.6.4 Percentage relative standard deviation (%RSD):

It is also known as coefficient of variation CV. It is defined as the standard deviation (S.D) expressed as the percentage of mean.

$$\mathbf{C}V \text{ or } \% \text{ RSD} = \frac{S.D}{\bar{x}} \times 100$$

Where, S.D = standard deviation,

$\bar{x}$ = Mean or arithmetic average.

The variance is defined as S^2 and is more important in statistics than S itself. However, the latter is much more commonly used with chemical data.

1.6.5 Standard Error of mean (S.E.):

Standard error of mean can be defined as the value obtained by division of standard deviation by square root of number of observations. It is mathematically expressed as $S.E. = \frac{S.D.}{\sqrt{n}}$

Where, n = number of observations.

S.D = Standard deviation

1.7 Data elements required for assay validation:

It is not always necessary to evaluate every analytical performance parameter, as different test methods require different validation schemes. The Most common categories of assays for which validation data should be required are as follows:

i) Quantitation of major components or active ingredients.

ii) Determination of impurities or degradation compounds.

iii) Determination of performance characteristics

Category-I: Analytical methods for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

Category-II: Analytical methods for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These methods includes quantitative assays and limit tests.

Category-III: Analytical methods for determination of performance characteristics (e.g. dissolution, drug release).

The type of method and its intended use dictates which parameters are required to be investigated. They are illustrated in the following Table 6.

Table 6: Data elements required for assay validation

Analytical Performance Parameter	Assay Category-I	Assay category-II		Assay Category-III
		Quantitative	Limit Test	
Accuracy	Yes	Yes	*	*
Precision	Yes	Yes	No	Yes
Specificity	Yes	Yes	Yes	*
LOD	No	No	Yes	*
LOQ	No	Yes	No	*
Linearity & range	Yes	Yes	No	*
Ruggedness	Yes	Yes	Yes	*

*may be required, depending on the nature of specific test.

Table 7: Comparison of Analytical Parameters Required for Assay validation

FDA reviewer Guidance	USP General Chapter <1225>	ICH Q2A Guidelines
Accuracy	Accuracy	Accuracy
Precision Repeatability Analysis	Precision	Precision
Intermediate precision	No	Intermediate precision
Reproducibility	No	No

FDA reviewer Guidance	**USP General Chapter <1225>**	**ICH Q2A Guidelines**
Specificity /selectivity	Specificity	Specificity
Detection limit	Detection limit	Detection limit
Quantitation limit	Quantitation limit	Quantitation limit
Linearity	Linearity	Linearity
Range	Range	Range
No	Ruggedness	No
Robustness	Robustness	Robustness
System suitability sample solution stability	System suitability	System suitability

2.0 LITERATURE REVIEW

2.1 Suneetha and Raja Rajeswari, IJPSR, 2015; Vol. 6(5): 1915-1923., Estimation of Teriflunomide Along with Concomitant Drugs in Different Biological Matrices Using LC-MS/MS. Int J Pharm Sci Res 2015; 6(5): 1915-23.doi: 10.13040/IJPSR.0975-8232.6(5).1915-23.

The proposed validated method for the estimation of teriflunomide in different biological matrices is highly sensitive and rapid compared to published reports. The method offers significant advantages over those previously reported, in terms of lower sample requirements, simplicity of extraction procedure without any matrix effect. The efficiency of protein precipitation extraction and without any interference from the concomitant drugs make it an attractive procedure in bio analysis of teriflunomide.

The linear dynamic range established was adequate to measure the concentration of teriflunomide in any preclinical and clinical study involving different biological species. The concomitant drugs also can be estimated along with the target analyte which is more advantageous than single compound analysis and also useful in drug interaction and toxicology studies.

2.2 Govind J. Kher, Vijay R. Ram, Kapil L. Dubal, Atul H. Bapodara and Hitendra. S. Joshi, Validation of a Stability-Indicating LC Method for Assay of Leflunomide in Tablets and for Determination of Content Uniformity. International Journal of ChemTech Research CODEN(USA): IJCRGG ISSN : 0974-4290, Vol. 3, No.2, pp 523-530, April-June 2011.

In the present work, an analytical method based on LC using UV detection was developed and validated for assay and determination of content uniformity of leflunomide in tablet dosage forms. The analytical conditions were selected after testing the different parameters that effect LC analysis, such as column, diluent, buffers, buffer concentration, organic solvent for mobile phase, proportion of mobile phase and concentration of analyte etc. The Wakosil column used because of its advantages of high retention, high resolving capacity, better reproducibility, low back pressure and low tailing. Our preliminary trials using different composition of mobile phases consisting of water with methanol or acetonitrile, did gave poor peak shape. By using 0.02M ammonium acetate buffer per 1000 mL and keeping mobile phase composition as of 0.02M ammonium acetate buffer and acetonitrile (40:60, v/v), best peak shape was obtained. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. A detection wavelength of 260 nm was selected after scanning the standard solution over the range 190-350 nm by using photo-diode array (PDA) detector. Detection at 260 nm resulted in good response and good linearity.

The drug substance was easily extracted from the pharmaceutical dosage form by use of acetonitrile and buffer 50:50 (v/v). The tablet dispersed readily in buffer and the drug substance was freely soluble in acetonitrile. Solutions of

standard and test preparations were found to be stable in this solvent mixture. After developing the analytical method, it was validated. The analytical method validation gave evidence that the procedure was suitable for the intended purpose. The analytical method validation was carried out as per guidelines of ICH Q2 (R1), USP and AOAC INTERNATIONAL.

2.3 Duygu Yeniceli, Dilek Dogrukol-Ak, , Muzaffer Tuncel. Department of Analytical Chemistry, Faculty of Pharmacy, Anadolu University, 26470 Eskisehir, Turkey. Determination of leflunomide in tablets by high performance liquid chromatography. Received 24 January 2005, Revised 24 June 2005, Accepted 28 June 2005, Available online 19 August 2005

In the present study, a reverse phase high performance liquid chromatography (HPLC) method was validated and applied for the determination of leflunomide in tablets. Chromatographic separation of leflunomide and oxazepam as an internal standard was carried out on a C18 column (50 mm, 3 mm i.d.) using a mobile phase, consisting of methanol and water (60:40, v/v), at a flow rate of 0.5 ml min−1 and UV detection at 260 nm. The retention times for oxazepam and leflunomide were 2.6 and 5.2 min, respectively. The validated quantification range of the method was 2.7×10^{-6} to 5.5×10^{-5} M for leflunomide. The results of the developed procedure in tablets were compared with those of UV spectrophotometry to assess active leflunomide content.

2.4 Balraj Saini, Gulshan Bansal. Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala 147002, India. Isolation and characterization of a degradation product in leflunomide and a validated selective stability-indicating HPLC–UV method for their quantification. Received 14 May 2014, Revised 26 September 2014, Accepted 10 October 2014, Available online 22 October 2014

Leflunomide (LLM) is subjected to forced degradation under conditions of hydrolysis, oxidation, dry heat, and photolysis as recommended by International Conference on Harmonization guideline Q1A(R2). In total, four degradation products (I–IV) were formed under different conditions. Products I, II and IV were formed in alkaline hydrolytic, acidic hydrolytic and alkaline photolytic conditions. LLM and all degradation products were optimally resolved by gradient elution over a C18 column. The major degradation product (IV) formed in hydrolytic alkaline conditions was isolated through column chromatography. Based on its 1H NMR, IR and mass spectral data, it was characterized as a British Pharmacopoeial impurity B. The HPLC method was found to be linear, accurate, precise, sensitive, specific, rugged and robust for quantification of LLM as well as product IV. Finally, the method was applied to stability testing of the commercially available LLM tablets.

2.5 D.S. Shokry, S.A. Weshahy Faculty of pharmaceutical sciences & pharmaceutical industries and M. Abdel Kawy Future University in Egypt. Faculty of Pharmacy, Cairo University. Application of Spectrophotometric and Chromatographic Methods for Stability Indicating Determination of Leflunomide. Journal of Applied Sciences Research, 8(3): 1547-1557, 2012 ISSN 1819-544X

Leflunomide, an amide containing compound, pyrimidine synthesis inhibitor a leading drug in treatment of moderate to severe rheumatoid arthritis.Four stability indicating methods are presented in this paper for the selective determination of Leflunomide in presence of its alkaline degradate. The resolution of the drug and its alkaline degradate has been achieved using derivative spectrophotometry including second-, third- and fourth derivatives. The derivative amplitudes are measured at 256.4 nm, 269.8 nm, 226.6 nm for D2, D3 and D4 respectively. The proposed methods were found to be linear over the range of (2.0-24.0 μgml-1). The fourth method is based on the chromatographic separation on a C18 column using a mobile phase of 0.01 M aqueous potassium dihydrogen phosphate adjusted at pH 3.5 with orthophosphoric acid, acetonitrile in a ratio of [30:70], a flow rate of 1 ml/min and UV detection at 262 nm. The proposed methods were validated with regard to accuracy, precision, selectivity, robustness, application to pharmaceutical preparation and further validated by applying standard addition technique.

3.0 AIM AND OBJECTIVE

The drug analysis plays an important role in the development, manufacture and therapeutic use of drugs. Most of the pharmaceutical industries do the quantitative chemical analysis to ensure that the raw material used and the final product thus obtained meet certain specification and to determine how much of each components are present in the final product. Standard analytical procedure for newer drugs or formulation may not be available in Pharmacopoeias; hence it is essential to develop newer analytical methods which are accurate, precise, specific, linear, simple and rapid.

Aim: To develop and validate new RP HPLC method for the assay of Teriflunomide in tablet dosage.

Objective: Literature survey has revealed that various method were reported for estimation of Teriflunomide. The existing methods are inadequate to meet the requirements; hence it is proposed to improve the existing methods and to develop new methods for the Estimation of Teriflunomide in pharmaceutical dosage forms. Hence, on the basis of literature survey it was thought to develop a precise, accurate, simple and reliable, less time consuming method for estimation.

4.0 PLAN OF WORK

Plan of Work

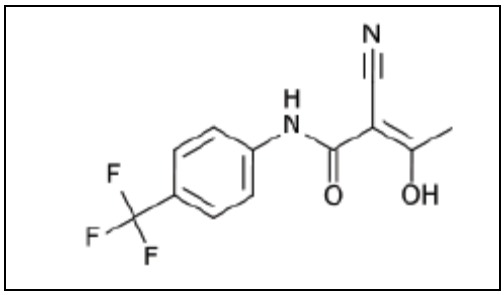
- Gather/ generate background information obtain physico-chemical properties.
- Determine if special handling/treatment of sample is needed.
- From physicochemical properties select detector parameters.
- Calculate approximately separation parameters/isocratic or gradient mode.
- Perform forced degradation experiments to challenge method.
- Optimization separation conditions
- Summarize methodology and finalize documentation.
- Analysis of marketed formulations and Validate method.

5.0 DRUG PROFILE

Proper Name : Teriflunomide

Synonyms : Teriflunomide

Structure :



Molecular formula : $C_{12}H_9F_3N_2O_2$

IUPAC name : (Z)-2-Cyano-3-hydroxy-but-2-enoic acid-(4-trifluromethyl phenyl)-amide
(or)
2-Cyano-3-hydroxy-N-[4-(trifluromethyl)phenyl]-2(Z)-butenamide

Molecular weight : 270.21 g/mol

CAS No. : 163451818

Melting point : 229 - 232°C

Description : White to almost white powder

Solubility : Sparingly soluble in acetone; slightly soluble in methylene chloride; very slightly soluble in acetonitrile; insoluble in water, ethanol and isopropyl alcohol.

pH (1% in water) : 3.19

Storage : Store at controlled room temperature, 15 to 30°C.

Category : Teriflunomide is the active metabolite of leflunomide, and it acts as an immunomodulatory agent by inhibiting pyrimidine synthesis.

Pharmacokinetic Data:

Teriflunomide is the principal active metabolite of leflunomide and is responsible for leflunomide's activity in vivo. At recommended doses, teriflunomide and leflunomide result in a similar range of plasma concentrations of teriflunomide.

Absorption :

After oral administration of teriflunomide, maximum plasma concentrations are reached, on average, in 14 hours.

Distribution:

After a single intravenous dose, the volume of distribution is 11 L.

Metabolism:

Teriflunomide mainly undergoes hydrolysis to minor metabolites. Other minor metabolic pathways include oxidation, Nacetylation and sulfate conjugation. Teriflunomide is not metabolized by CYP450 or flavin monoamine oxidase.

Elimination:

Teriflunomide is eliminated unchanged and mainly through bile. Specifically 37.5% is eliminated in the feces and 22.6% in urine.

Indications:

Used in the treatment of relapsing forms of multiple sclerosis (MS).

Pharmacology:

Teriflunomide is an immunomodulatory agent that decreases the amount of activated CNS lymphocytes, which results in anti-inflammatory and antiproliferative effects.

Mechanism of Action:

The exact mechanism by which teriflunomide acts in MS is not known. What is known is that teriflunomide prevents pyrimidine synthesis by inhibiting the

itochondrial enzyme dihydroorotate dehydrogenase, and this may be involved in its immunomodulatory effect in MS.

Adverse Effects:

Most common adverse reactions (≥10% and ≥2% greater than placebo): ALT increased, alopecia, diarrhea, influenza, nausea, paresthesia and warning for Hepatotoxicity and risk of Teratogenicity

Hepatotoxicity Severe liver injury including fatal liver failure has been reported in patients treated with leflunomide, which is indicated for rheumatoid arthritis.

Risk of Teratogenicity Based on animal data, Teriflunomide may cause major birth defects if used during pregnancy.

Toxicity:

Teriflunomide is contraindicated in pregnant women or women of childbearing age due to the risk of teratogenicity. Teriflunomide is also contraindicated in severe hepatic impairment due to reports of hepatotoxicity, hepatic failure, and death.

6.0 MATERIALS AND METHODS

Table 8: Instrumentation

S.NO	Instrument Name	Make	Model
1.	HPLC	Waters	2996 PDA
2.	Semi Micro Balance	Sartorius	CPA225D
3.	Micro Balance	Sartorius	CPA2P
4.	pH meter	Thermo Electron Corporation	Orion 3 Star
5.	Sonicator	Spectra lab	UCB70
6.	Centrifuge Apparatus	Hermle centrifuge apparatus	Not Available
7.	UV Visible Spectrophotometer	Shimazdu	UV-1700

Table 9: Reagents and Chemicals

S.No.	Chemicals/Reagents	Make/Grade
1.	Acetonitrile	Merck, (HPLC-Grade)
2.	Potassium dihydrogen orthophosphate	Merck (GR-Grade)
3.	Ortho phosphoric acid	Merck (GR-Grade)
4.	Potassium Hydroxide	Merck (GR-Grade)
5.	Ammonium acetate	Merck (GR-Grade)
6.	Water	Milli-Q Water

Table: 10 Filters Used

S.No.	Name of the filter
1.	0.45µm PVDF membrane filter (Manufactured by PALL)
2.	0.45µm NYLON membrane filter (Manufactured by PALL)

Table: 11 Working/reference standards

S.No	Name of Working/reference standards
1.	Teriflunomide Working Standard (Manufactured by Glenmark)

Table: 12 Test Sample

S.No	Name of Test Sample
1.	Teriflunomide Tablets, 14mg (Manufactured by Dr.Reddys)

Table: 13 Column Used

S.No	Column Description
1.	X Bridge C8 column (150 x 4.6 mm, 5 µm particle size)
2.	Zorbax Eclipse XDB C8 column (150 x 4.6 mm, 5 µm particle size)

METHOD DEVELOPMENT

The objective of this experiment was to optimize the assay method for estimation of Teriflunomide tablets based on the literature survey and the trails made. The trials mentioned bellow describes how the optimization was done.

Trial 1:

Buffer preparation	**:**	50Mm of Ammonium Acetate buffer, filter through 0.45µm membrane filter and degas.
Mobile Phase	**:**	Buffer and ACN (60:40). Sonicated to degas.
Diluent	**:**	Water : ACN (30 : 70)

Chromatographic conditions:

Column	**:**	X Bridge C8 column (150 x 4.6 mm, 5 µm particle size)
Column temperature	**:**	30°C
Sample temperature	**:**	5°C
Elution mode	**:**	Isocratic
Flow rate	**:**	1.0 ml/min
Injection volume	**:**	10µl
Detector wave length	**:**	294nm
Run time	**:**	10 min.
Seal wash	**:**	90:10 (Water: ACN)
Needle wash	**:**	10:90 (Water: ACN)
System Suitability	**:**	USP Tailing Factor – NMT 2.0 and Plate count - NLT 2000.

Conclusion : The peak was observed but the Plate count is below 2000.

Trial 2:

Buffer preparation : Similar to Trial 1

Mobile Phase : Similar to Trial 1

Diluent : Similar to Trial 1

Chromatographic conditions:

Column : Zorbax Eclipse XDB C8 column (150 x 4.6 mm, 5 µm particle size)

Column temperature : 30°C

Sample temperature : 5°C

Elution mode : Isocratic

Flow rate : 1.0 ml/min

Injection volume : 10µl

Detector wave length : 294nm

Run time : 10 min.

Seal wash : 90:10 (Water: ACN)

Needle wash : 10:90 (Water: ACN)

System Suitability : USP Tailing Factor – NMT 2.0 and Plate count - NLT 2000.

Conclusion : The peak was observed but the Plate count is below 2000 and peak shape was asymmetrical.

Trial 3 (Optimized Method):

Buffer preparation	**:**	20Mm of Potassium dihydrogen orthophosphate buffer pH 2.40, filter through 0.45µm membrane filter and degas.
Mobile Phase	**:**	Buffer and ACN (65:35). Sonicated to degas.
Diluent	**:**	Water : ACN (30 : 70)

Chromatographic conditions:

Column	**:**	Zorbax Eclipse XDB C8 column (150 x 4.6 mm, 5 µm particle size)
Column temperature	**:**	30°C
Sample temperature	**:**	5°C
Elution mode	**:**	Isocratic
Flow rate	**:**	1.0 ml/min
Injection volume	**:**	10µl
Detector wave length	**:**	250nm
Run time	**:**	10 min.
Seal wash	**:**	90:10 (Water: ACN)
Needle wash	**:**	10:90 (Water: ACN)
System Suitability	**:**	USP Tailing Factor – NMT 2.0 and Plate count - NLT 2000.
Conclusion	**:**	The peak was observed with good tailing and good shape, with plate count above 2000 (10721) and tailing factor below 2 (1.3). And this method was finalized for assay of Teriflunomide Tablets.

Preparation of Standard solution:

Weigh accurately about 50 mg of Teriflunomide RS/WRS and transfer to a 200 mL volumetric flask. Add 140 mL of diluent and sonicate to dissolve. Dilute to volume with diluent and mix well.

Transfer 10 mL of standard stock preparation into a 50 mL volumetric flask. Dilute to volume with diluent and mix well. (Concentration of about 50 µg/mL of Teriflunomide).

Preparation of Sample solution:

Determine the Average weight using not less than 20 tablets. Weigh and finely powder not less than 20 tablets. Weigh accurately and transfer tablet powder equivalent to about 25 mg into a 100 mL volumetric flask. Add 70 mL of diluent and sonicate for 30 minutes with intermittent shaking. Dilute to volume with diluent and mix well. Centrifuge a portion of the above solution at 3500 rpm for 10 minutes.

Transfer 5 mL of the supernatant solution to a 25 mL volumetric flask, dilute to volume with diluent and mix well.

Filter a portion of the above solution through a 0.45 µm PVDF filter after discarding atleast the first 4 mL of the filtrate.

(Sample preparation, concentration of about 50 µg/mL of Teriflunomide).

Procedure:

Equilibrate the column with mobile phase for not less than 30min at a flow rate of 1.0 l/min. Separately inject 10 µl of Blank (diluent), Standard solution (five times) and Sample solution into the chromatographic system. Record the chromatograms and measure the peak responses.

System suitability:

The column efficiency as determined for the Teriflunomide peak from standard solution is NLT 2000 theoretical plates.

Tailing factor for Teriflunomide peak obtained from standard chromatogram should be NMT 2.0

The % RSD for the Teriflunomide peak for 5 replicate injections of standard solution should be NMT 2.0

The retention time of Teriflunomide peak is about 6.0 minutes.

Calculations:

Quantity of Teriflunomide Present in the tablet as % of labelled amount:

$$= \frac{A_T}{A_S} \times \frac{Ws}{200} \times \frac{10}{50} \times \frac{100}{W_T} \times \frac{25}{5} \times \frac{P}{100} \times \frac{AW}{L} \times 100$$

A_T : Peak area of Teriflunomide from the chromatogram of the assay preparation

A_S : Mean peak area of Teriflunomide from the chromatogram of the standard preparation.

Ws : Weight of Teriflunomide working standard taken, in mg

W_T : Weight of tablet powder taken, in mg

P : Potency of Teriflunomide working standard used in percent on as is basis

L : Label claim in mg

AV : Average weight of tablet in mg

7.0 RESULTS AND DISCUSSION

7.1 System Suitability:

A Standard solution was prepared by using Teriflunomide working standards as per test method and was injected five times into the HPLC system.

The system suitability parameters were evaluated from standard chromatograms.

Acceptance criteria:

1. The USP Tailing factor should be not more than 2.0 for Teriflunomide peak from standard solution.
2. The USP Theoretical plate count should be not less than 2000 for Teriflunomide peak from standard solution.
3. The RSD of Teriflunomide peak area is NMT 2.0% from five replicate injections of standard solution.

Table 14 System Suitability

Injection	Peak Area	USP Plate count	USP Tailing
1	1616310	7147	1.27
2	1617462	7192	1.28
3	1621285	7096	1.28
4	1618228	7215	1.27
5	1610144	7220	1.28
SD	1616686	---	---
% RSD	0.25	---	---

Observation:

The %RSD for peak areas, USP plate count and USP tailing were found to be within the limits.

7.2 Accuracy: (Recovery):

A study of Accuracy was conducted. Drug Assay was performed in triplicate as per test method by spiking the Teriflunomide drug substance to the placebo equivalent to 5%, 50%, 100% and 200% of the labeled amount as per the test method. The average % recovery of Teriflunomide was calculated.

Separately inject the blank, placebo, Teriflunomide in to the chromatograph.

Acceptance criteria:

The mean % recovery of, Teriflunomide the at each level should be not less than 95.0% and not more than 105.0%.

Table 15 Accuracy

Sample No.	Theoretical (%)	Mean Peak area	% Recovery	Mean (%) Recovery	% RSD
1	5	79271	99.37	99.63	0.45
2	5	79243	99.37		
3	5	79893	100.15		
1	50	784059	100.65	100.44	0.21
2	50	786797	100.22		
3	50	789985	100.46		
1	100	1589177	101.16	101.05	0.28
2	100	1587887	100.73		
3	100	1593464	101.28		
1	200	3172132	101.92	101.15	0.73
2	200	3155725	101.12		
3	200	3111902	100.43		

Observation:

The recovery results indicating that the test method has an acceptable level of accuracy. Therefore the method is accurate.

7.3 Precision:

A. System precision: Standard solution was prepared as per test method and injected five times into chromatographic system.

B. Method precision: Prepared six sample solutions as per test method and injected each solution into chromatographic system.

Acceptance criteria:

1. %RSD of %assay results from six samples should be NMT 2.0

2. Assay should be in the range of test method i.e. not less than 95.0% and not more than 105.0%.

a) System Precision:

Table 16 System Precision

	Injection	Peak Areas	Theoretical plates	Tailing factor
Concentration 100%	1	1616310	7147	1.27
	2	1617462	7192	1.28
	3	1621285	7096	1.28
	4	1618228	7215	1.27
	5	1610144	7220	1.28
Statistical Analysis	**Mean**	1616686	---	---
	SD	1616686	---	---
	% RSD	0.25	---	---

b) Method precision:

Table 17 Method Precision

Sample No.	Area	%Assay
1.	1570314	**97.87**
2.	1574009	**98.18**
3.	1594849	**99.27**
4.	1590749	**99.07**
5.	1606478	**100.12**
6.	1609080	**100.31**
Mean		**99.13**
Standard Deviation		**0.98848**
% RSD		**0.99**

Observation:

The precision study has shown that the test method is precise.

7.4 Linearity of test method:

Seven linearity solutions were prepared using Teriflunomide working standard at concentration levels from 5% to 150% of target concentration of Teriflunomide (5%, 10%, 25%, 50%, 75%, 100% and 150%). The linearity graph plotted from 5% to 150%.

Acceptance criteria:

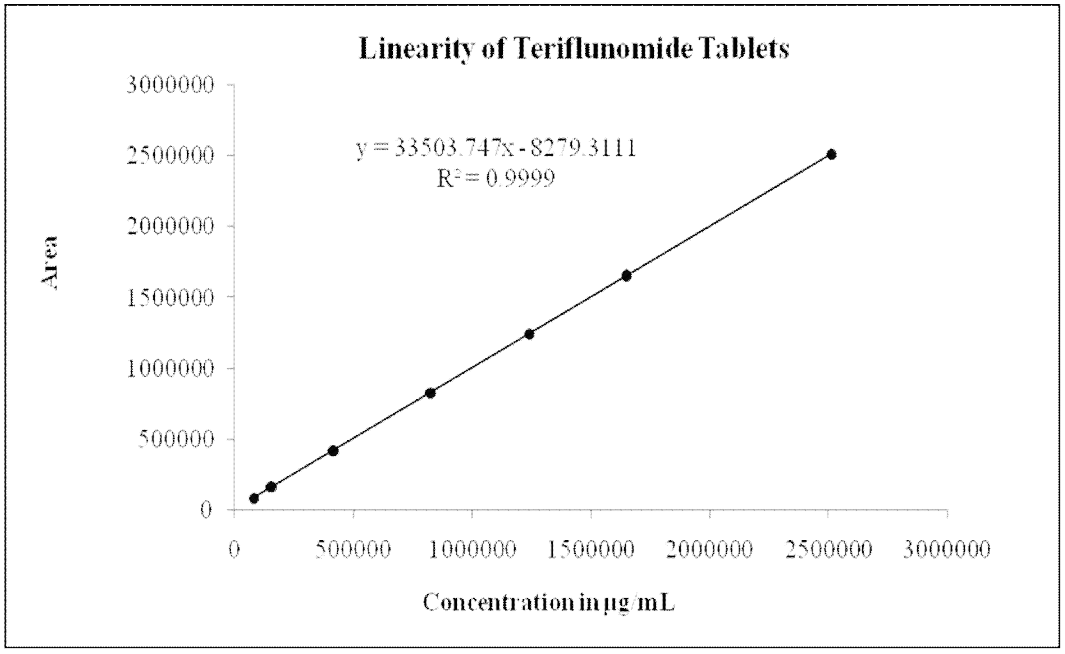
Correlation Coefficient should be not less than 0.999.

% of RSD for level 1 and Level 5 should be not more than 2.0.

Table 18 Linearity

Linearity Level	Concentration (µg/mL)	Average Area
L1-5%	2.502	83096
L2-10%	5.004	154525
L3-25%	12.512	414647
L4-50%	25.024	823580
L5-75%	37.537	1241003
L6-100%	50.049	1650624
L7-150%	75.074	2510914

Linear Regression Analysis	Concentration in µg/mL vs. Area
Correlation Coefficient Square (r^2)	0.9999
Slope	33503.747
Y-Intercept	-8279.311



Observation:

The correlation coefficient was found to be 0.9999.

From the above study it was established that the linearity of test method is from 5% to 150% of the target concentration.

Range:

Data from linearity, precision, accuracy sections was considered to establish range of the method. The results were summarized in table 19.

Acceptance Criteria:

For linearity, correlation coefficient shall be ≥ 0.999.

For precision, %RSD of assay of 6 replicate sample preparations shall be NMT 2.0%.

For accuracy, individual recovery at each spike level should be within 95.0% to 105.0%

Table 19 Range

Parameter	Acceptance Criteria	Result
Linearity	R ≥ 0.999	0.999
Precision	%RSD of 6 Replicates NMT 2.0	0.99
Accuracy	Recovery 95.0% to 105.0%	99.99

7.5 Selectivity/Specificity:

i) Placebo and impurity interference:

A study to establish the interference of placebo was conducted. Blank, Standard, Placebo, Sample solution were prepared and injected into the chromatographic system for Identification and interference with the Teriflunomide peak.

Acceptance Criteria:

a. No interference should be observed from diluent, individual impurity and placebo at the retention time of Teriflunomide Peak.

b. Teriflunomide Peak should be separated from the known and unknown impurities peak. (USP resolution NLT 1.5)

Table 20 Placebo interference

Sample Name	Retention Time (minutes)	Interference
Blank	Not Detected	Nil
Standard	NA	NA
Placebo	Not Detected	NA
Control Sample	NA	NA
Spiked Sample	NA	NA
Impurity A	2.597	Nil
Impurity B	6.295	Nil
Impurity C	19.041	Nil
Impurity D	18.111	Nil

Observation:

From the chromatograms, it was concluded that there was no interference with placebo as no peaks were observed at the retention times of Teriflunomide peaks.

ii) Interference from degradation products:

A study was conducted to demonstrate the effective separation of degradants from Teriflunomide. Separate portions of Drug product and Placebo were exposed to following stress conditions to induce degradation.

a) Acid stress

b) Base stress

c) Peroxide stress

d) UV light stress

e) Heat stress

f) A sample solution was prepared as per the method.

Acid Stress Sample:

Weighed accurately and transferred Teriflunomide tablet powder equivalent to 25 mg of Teriflunomide into a 100 mL volumetric flask. Added 35 mL of RS-diluent and sonicated for 30 minutes with intermittent shaking. 3mL of 1N HCL was added and heated on a water bath at 80°C for 3 hours. Cooled to room temperature and neutralized with 3 mL of 1N NaOH. Diluted to volume with RS-diluent and mixed well. Centrifuge a portion of the above solution at 3500 rpm for 10 minutes and further 5 mL of supernatant solution was transferred to 50 mL flask and diluted to volume with assay diluent and mixed well. Filtered through 0.45 µm PVDF filter by discarding the first 4 mL of the filtrate.

Base Stress Sample:

Weighed accurately and transferred Teriflunomide tablet powder equivalent to 25 mg of Teriflunomide into a 100 mL volumetric flask. Added 35 mL of RS-diluent and sonicated for 20 minutes with intermittent shaking. 3 mL of 1N NaOH was added and

heated on a water bath at 80°C for 8 hours. Cooled to room temperature and neutralized with 3 mL of 1 N HCl. Diluted to volume with RS-diluent and mixed well. Centrifuged a portion of the above solution at 3500 rpm for 10 minutes and further 5 mL of supernatant solution was transferred to 50 mL flask and diluted to volume with assay diluent and mixed well. Filtered through 0.45 µm PVDF filter by discarding the first 4 mL of the filtrate.

Peroxide Stress Sample:

Weighed accurately and transferred Teriflunomide tablet powder equivalent to 25 mg of Teriflunomide into a 100 mL volumetric flask. Added 20 mL of RS-diluent and sonicated for 30 minutes with intermittent shaking. 3 mL of 30% hydrogen peroxide was added and heated on a water bath at 80°C for 30 minutes. Cooled to room temperature, diluted to volume with RS-diluent and mixed well. Centrifuged a portion of the above solution at 3500 rpm for 10 minutes and further 5 mL of supernatant solution was transferred to 50 mL flask and diluted to volume with assay diluent and mixed well. Filtered through 0.45 µm PVDF filter by discarding the first 4 mL of the filtrate.

UV Light Stress Sample:

Weigh accurately and transfer tablet powder equivalent to about 25 mg(Stressed under UV light for 24 hours) into a 100 mL volumetric flask. Add 70 mL of diluent and sonicate for 30 minutes with intermittent shaking. Dilute to volume with diluent and mix well. Centrifuge a portion of the above solution at 3500 rpm for 10 minutes.

Transfer 5 mL of the supernatant solution to a 25 mL volumetric flask, dilute to volume with diluent and mix well.

Filter a portion of the above solution through a 0.45 µm PVDF filter after discarding atleast the first 4 mL of the filtrate.

Heat Stress Sample:

Weigh accurately and transfer tablet powder equivalent to about 25 mg(Heated at 105°C for 1 hour and 30 minutes) into a 100 mL volumetric flask. Add 70 mL of diluent and sonicate for 30 minutes with intermittent shaking. Dilute to volume with diluent and mix well. Centrifuge a portion of the above solution at 3500 rpm for 10 minutes.

Transfer 5 mL of the supernatant solution to a 25 mL volumetric flask, dilute to volume with diluent and mix well.

Filter a portion of the above solution through a 0.45 µm PVDF filter after discarding atleast the first 4 mL of the filtrate.

Blanks were prepared in the similar way for Acid /Base reagent and for oxidation.

Placebo Solutions were prepared in the similar way for control, Acid, Base, Peroxide, heat and UV light degradation.

Acceptance Criteria:

The net degradation should be in between 1% to 50%.

All degradation Products should be separated from active.

Purity angle should be less than purity Threshold.

Table 21 Forced Degradation Studies

Sample Name	Condition	% Assay	% Degradation	Purity Angle	Purity Threshold
Control Sample	NA	99.77	NA	0.034	0.201
Spike Sample	NA	NA	NA	0.036	0.213
Acid Stress Sample	3 mL 5N HCl, heated on a water bath at 80°C for 3 hours.	78.97	20.80	0.034	0.202
Base Stress Sample	3 mL 0.1N NaOH, heated on a water bath at 60°C for 8 hours.	99.65	0.12	0.034	0.201
Peroxide Stress Sample	3 mL 30% H_2O_2, heated on a water bath at 80°C for 30 minutes.	84.75	15.02	0.033	0.204
UV light Stress Sample	Stressed under UV light for 24 hours.	100.77	NA	0.046	0.204
Heat Stress Sample	Heated in an oven at 105°C for 1 hour and 30 minutes.	98.90	0.87	0.044	0.203

Observation:

During the Specificity and forced degradation, it was observed that no secondary peak arising from degraded samples interfered with the elution of the Teriflunomide Peak. Analysis of blank, individual impurities and placebo preparations demonstrated no interference with Teriflunomide Peak elution. Peak purity analysis using the photodiode array detector demonstrated Teriflunomide Peak homogeneity. Known and unknown impurities well separated from main peak (Resolution NLT 1.5). The study validates that the method is specific and stability indicating.

7.6 Ruggedness of test method:

System to system /Analyst to Analyst/column to Column variability:

System to system /Analyst to Analyst/column to Column variability study was conducted on different HPLC systems, different columns and different analysts under similar conditions at different times. Six samples were prepared and each were analysed as per test method. The relative standard deviation for Teriflunomide was found to be below 2 % on the columns, systems and Analysts.

Comparison of both the results obtained on two different HPLC systems, different column and different analysts shows that the assay test method is rugged for System to system /Analyst to Analyst/column to Column variability.

Acceptance criteria:

1) The system suitability acceptance criteria as described in the method must be met.
2) The % RSD of Teriflunomide from the six sample preparations should be not more than 2.0%.
3) All assay values should be within the 90.0 – 110.0 % of label claim.

Table 22 System Suitability Ruggedness Results

Injection No.	Analyst-1	Analyst-2
	Peak area	Peak area
1	1616310	1546066
2	1617462	1556162
3	1621285	1552999
4	1618228	1555638
5	1610144	1555822
Mean	1616686	1553337
% RSD	0.25	0.27
Tailing factor	1.27	1.39
Plate count	7147	7861

Sample No	Analyst-1		Analyst-2	
	Mean Peak area	% Assay	Mean Peak area	% Assay
1	1570314	97.22	1533048	98.39
2	1574009	97.51	1531820	98.43
3	1594850	98.57	1543101	99.02
4	1590749	98.36	1529833	98.21
5	1606478	99.40	1536675	98.52
6	1609080	99.62	1529051	97.82
Mean	NA	98.44	NA	98.40
%RSD	NA	0.98	NA	0.4
Cumulative RSD of 12 samples assay value: 1.00 %				

Observation: The % of assay values obtained from six samples was between 90.0% and 110.0%. The RSD of assay from 6 samples is less than 2.0%.

7.7 Solution Stability

Establish the stability of standard and sample solution on bench top for a period of 2 days and in refrigerator for a period of about 5 to 7 days. Standard solution and sample solution were prepared as per test method injected and evaluated at initial, 12Hr, 24Hr and 48Hr.

The concentration of standard at 24 hours and 48 hours were compared to that of the initial. The assay of sample at 24 hours and 48 hours were compared to that of the initial.

Acceptance Criteria:

The concentration and % assay difference between the initial and time point for standard and sample solution should be NMT 2.0% from the initial value respectively.

Table 23 Solution stability of standard at 5°C

Time (hours)	Mean Peak area	Concentration (µg/mL)	% Difference from Initial
Initial	1616686	50.049	NA
24 hours	1624042	50.709	-1.31
48 hours	1633427	51.002	-1.90

Table 24 Solution stability of sample at 5°C

Time (hours)	Mean Peak area	Assay %	% Difference from Initial
Initial	1570315	97.22	NA
24 hours	1582351	98.81	-1.63
48 hours	1583676	98.89	-1.71

Observation:

The concentration of the 24 and 48 hours injections of standard solution differed by less than 2.0% when compared to the initial standard solution and assay value of the 24 hours and 48 hours of sample solution differed by less than 2.0% when compared to the initial sample solution. Therefore, the standard and sample solutions can be used up to 48 hours after its preparation if it is stored at 5°C.

7.8 Filter Study: (At least two filters)

For demonstrating that the filtration does not affect the analysis results. At least two types of filters were validated before use.

A sample was prepared as per the method for the filter study. This sample was divided into three portions. One portion of the prepared sample was centrifuged at 3500 RPM for 10 minutes. The centrifuged sample was used as a control for the filter study. Second portion of sample was filtered through 0.45µ PVDF filter and the filtrate was collected after discarding the first 3 mL, 4 mL, 5 mL and 6 mL of the filtrate. The third portion of sample was filtered through 0.45µ nylon filter and the filtrate was collected after discarding the first 3 mL, 4 mL, 5 mL and 6 mL of the filtrate. The centrifuged and filtered samples were injected.

Acceptance Criteria:

Compare the results of the filtered samples with that of the centrifuged sample preparation. Difference between peak area response of centrifuged sample and filtered sample should be not more than 2.0%.

Table 25 Filter study of 0.45µm PVDF and Nylon filter

Sample Name	Mean Peak area	% Difference
Centrifuged Sample (10 min @ 3500rpm)	1586029	NA
0.45µ PVDF filtrate sample, 3 mL discarded	1580351	0.35
0.45µ PVDF filtrate sample, 4 mL discarded	1582633	0.21
0.45µ PVDF filtrate sample, 5 mL discarded	1589423	-0.21
0.45µ PVDF filtrate sample, 6 mL discarded	1594893	-0.55
0.45µ Nylon filtrate sample, 3 mL discarded	1608774	-1.43
0.45µ Nylon filtrate sample, 4 mL discarded	1605951	-1.25
0.45µ Nylon filtrate sample, 5 mL discarded	1613098	-1.70
0.45µ Nylon filtrate sample, 6 mL discarded	1611165	-1.58

Observation:

The area found in the filtered fractions of sample solution was comparable to the area found in the centrifuged portion of the sample solution. There is no significant difference in area between different volumes 0.45µm PVDF and Nylon filtered. Therefore, the filters are suitable for use and the discarding of 4 mL of sample solution as filtrate, as stated in the method is a suitable volume to discard before collecting for analysis by HPLC.

7.9 Robustness:

The robustness is a measure of method capacity to remain unaffected by small, deliberate variations in method parameters and provides an indication of method reliability during normal use.

A standard solution was prepared and injected into the chromatographic system as per the conditions specified in the method. The same standard solution was re-injected by changing one parameter at a time, keeping other parameters constant. A set of system

suitability data was calculated for standards injected under altered method conditions and compared against the values generated under normal method conditions.

Method Parameters:

1. Flow Rate (Normal flow is 1.5 mL/min)

    a. Flow minus → 1.35 mL/min
    b. Flow plus → 1.65 mL/min

2. Column Operating Temperature (Normal temperature is 30°C)

    a. Temperature minus → 25° C
    b. Temperature plus → 35° C

3. Buffer pH variation (Normal Buffer pH 2.4)

    a. pH minus → pH 2.6
    b. pH plus → pH 2.2

4. Mobile Phase Composition Variation (Normal Composition is Buffer: ACN, 650:350)

    a. MPV1 → Buffer : ACN (620:380)
    b. MPV2 → Buffer : ACN (580:420)

Acceptance criteria:

All the system suitability requirements must be met.

Table 26 Robustness study - Comparison of System Suitability and Retention time

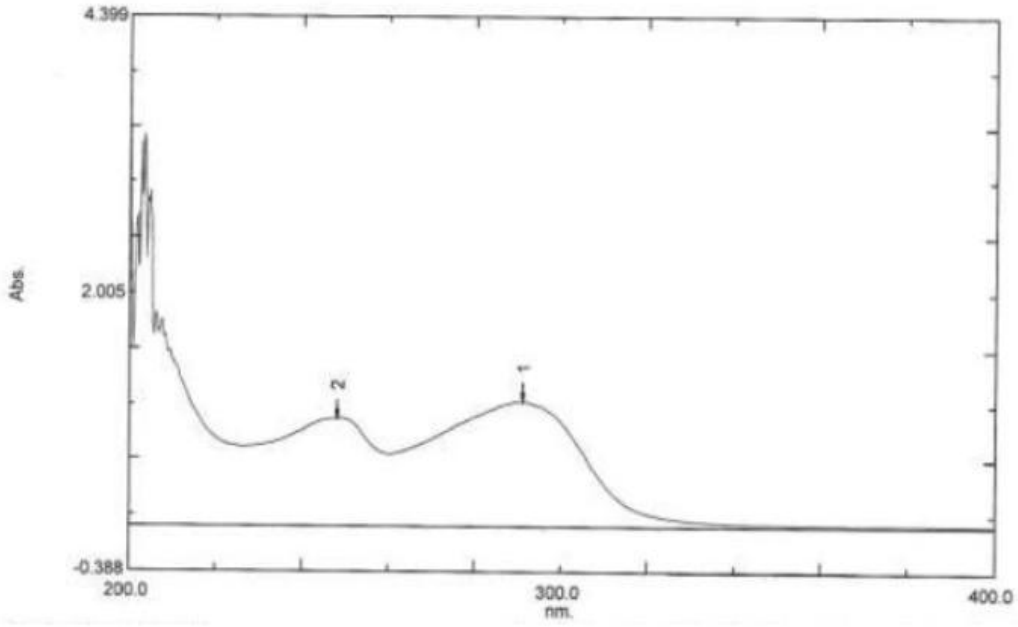
Parameters		Retention Time (min)	Mean Peak area (n=5)	%RSD	USP Tailing factor	USP Plate count
Normal Condition (1.0mL/min, 30°C, pH 2.4 Buffer : ACN (650:350)		6.564	1651509	0.61	1.25	7816
Flow Rate Minus	1.35 mL/min	7.292	1846396	0.23	1.26	8080
Flow Rate Plus	1.65 mL/min	5.996	1504834	0.35	1.24	7658
Mobile phase pH Minus	2.2	9.196	1585481	0.23	1.16	9241
Mobile phase pH Plus	2.6	5.683	1684507	0.21	1.33	6907
Column Temperature Minus	25°C	6.775	1677411	0.33	1.25	7756
Column Temperature Plus	35°C	6.144	1666426	0.09	1.25	8.35
Mobile Phase composition Variation 1	Buffer : ACN 670:330	9.215	1648619	0.17	1.23	8693
Mobile Phase composition Variation 2	Buffer:ACN 630:370	5.292	1665191	0.30	1.26	7259

Conclusion:

No significant change was observed in retention time after individually changing the conditions of flow rate of mobile phase, column operating temperature and pH of buffer and mobile phase composition variation. Calculations for all other system suitability parameters met the acceptance criteria and the data generated are comparable with the normal conditions. Based on the above result, it is concluded that the method is unaffected by small, deliberate variations in flow rate, column temperature and pH of buffer and mobile phase composition variation.

CHROMATOGRAMS

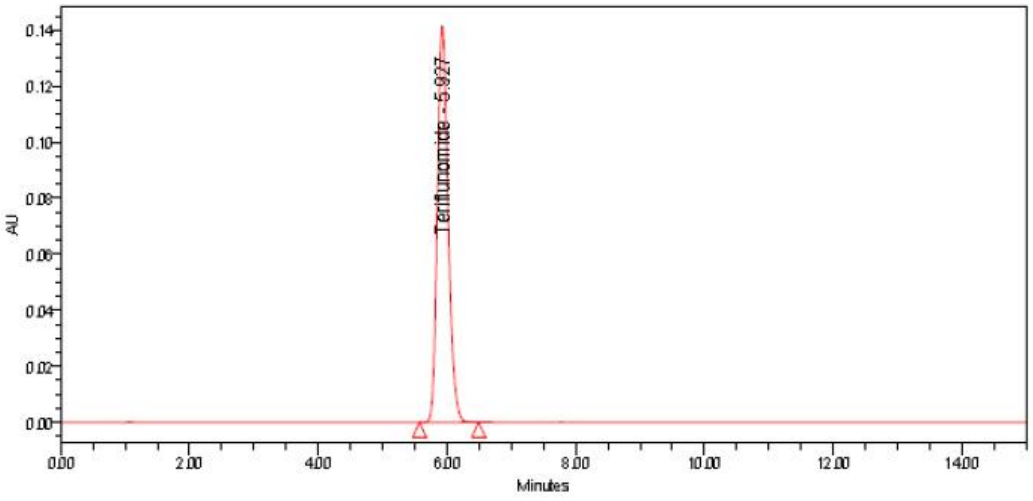
UV Spectrum



No.	P/V	Wavelength	Abs.	Description
1		291.0	1.065	
2		248.0	0.919	

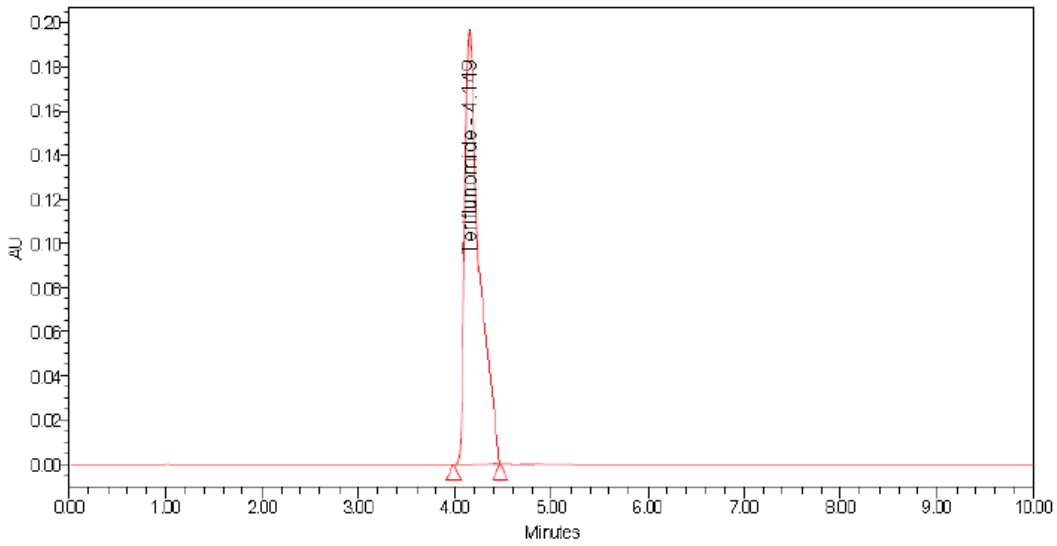
Method Development trails in HPLC

Trial No.1



	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	2	1	5.927	1610115	100.00	1950	1.23

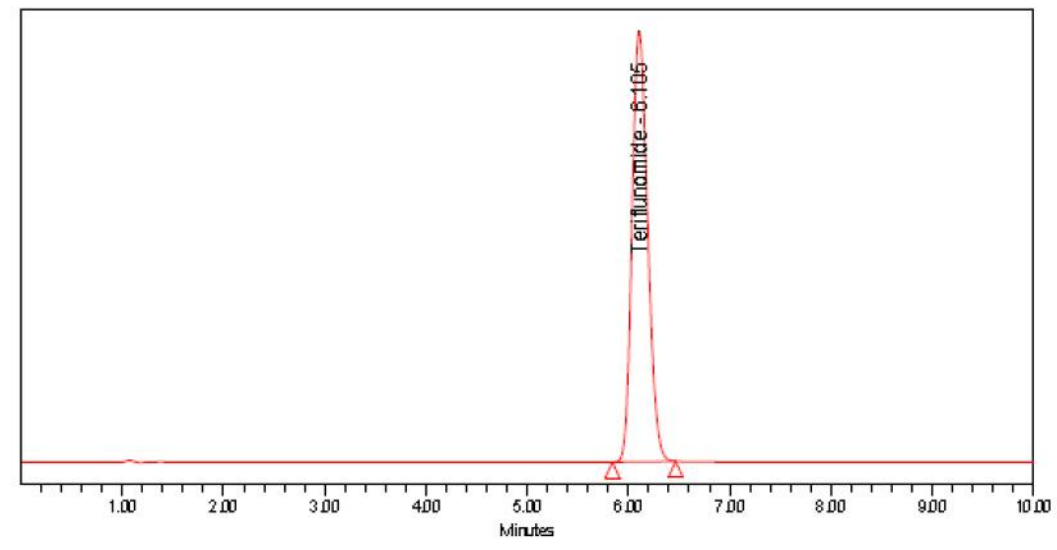
Trial No. 2



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	2	1	4.149	1569351	100.00	1903	1.73

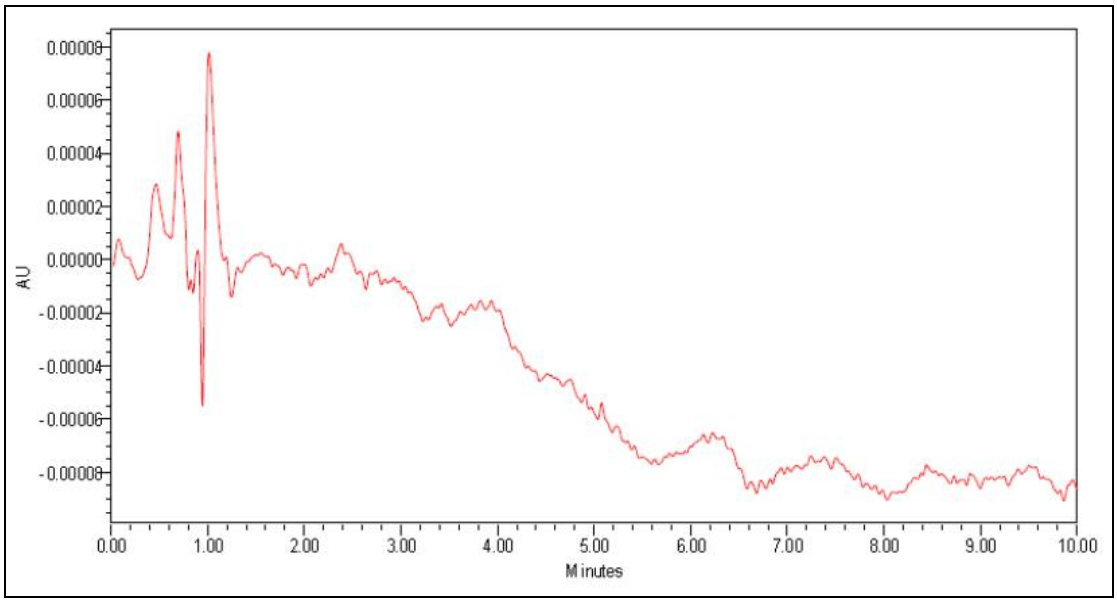
Trial No. 3 (Optimised method)



Peak Results

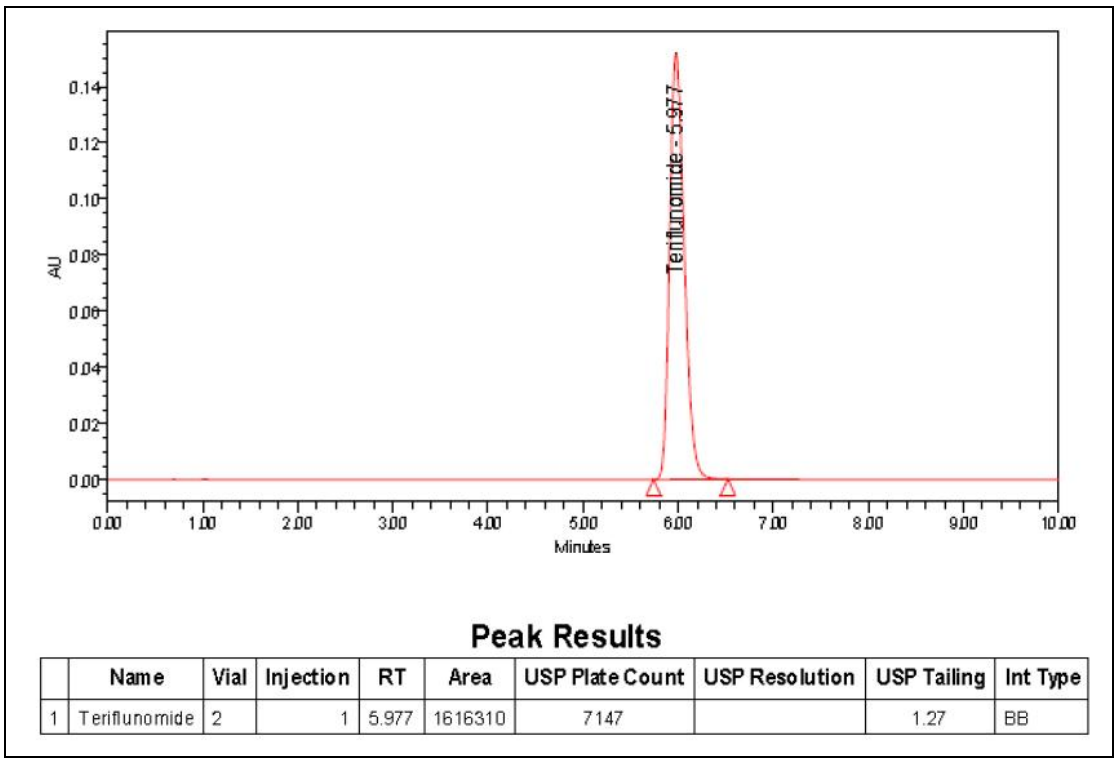
	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	2	1	6.105	1664517	7391		1.19	BB

Diluent Effect:



System Suitability

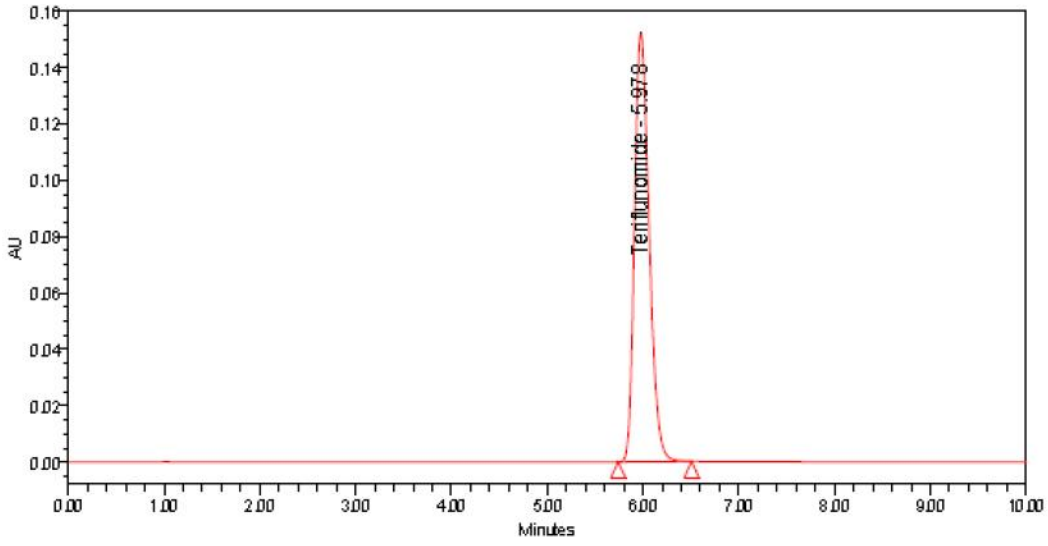
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	2	1	5.977	1616310	7147		1.27	BB

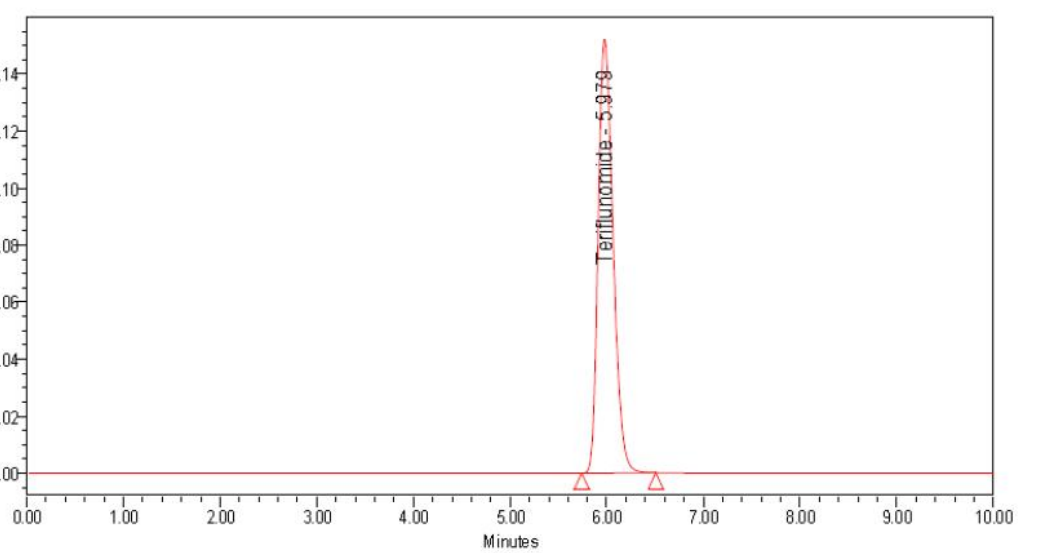
Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	2	2	5.978	1617462	7192		1.28	BB

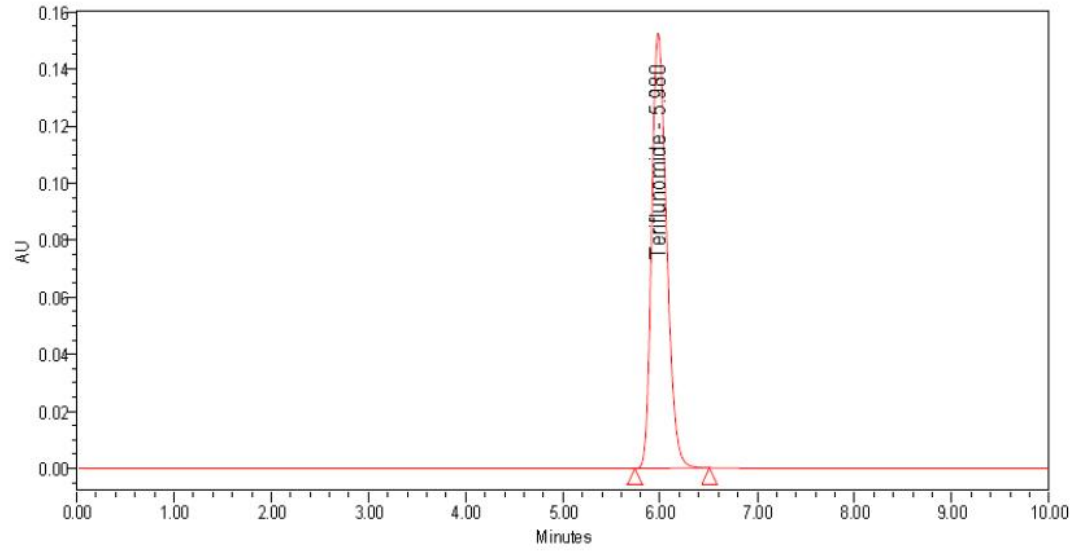
Injection-3



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	2	3	5.979	1621285	7096		1.28	BB

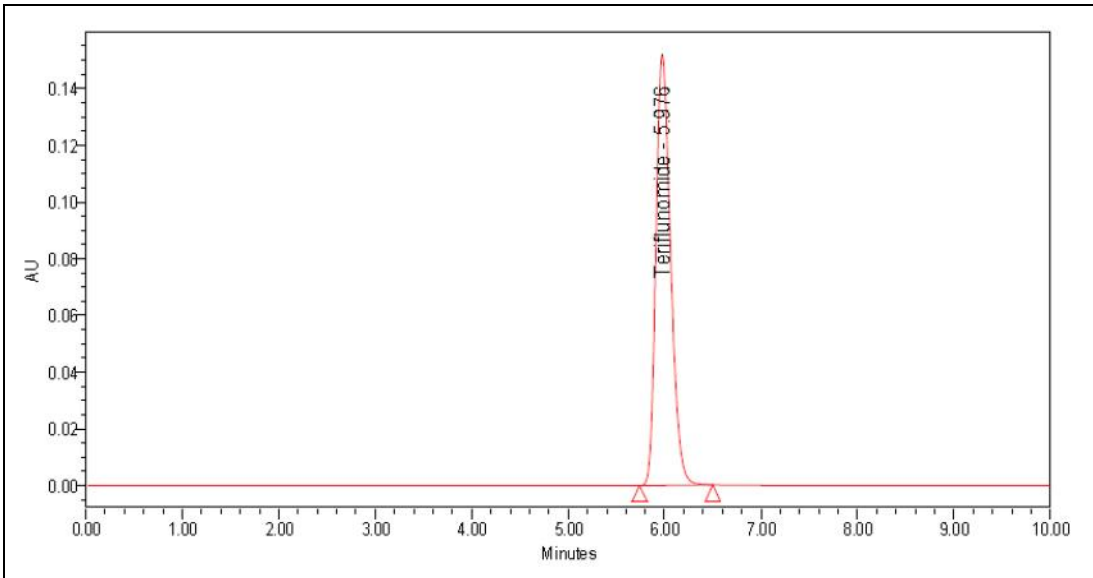
Injection-4



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	2	4	5.980	1618228	7215		1.27	BB

Injection-5

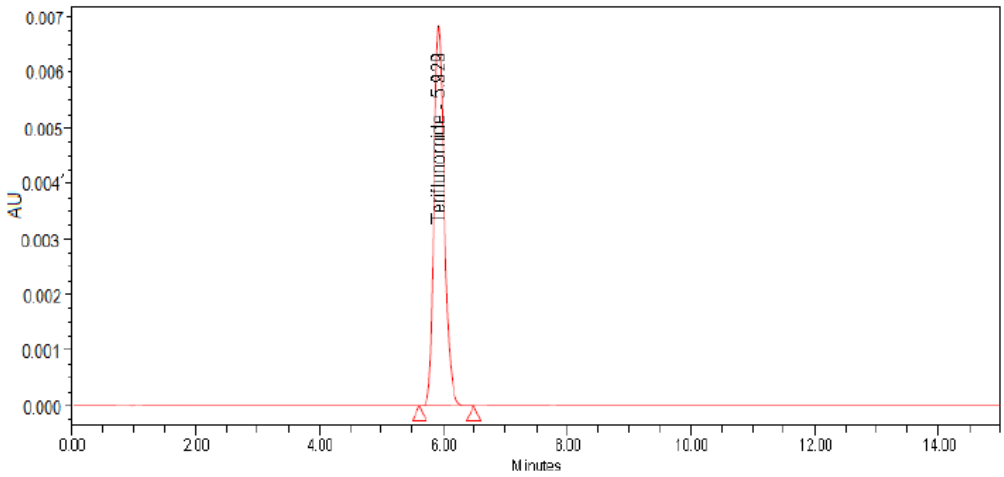


Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	2	5	5.976	1610144	7220		1.28	BB

Accuracy Chromatograms

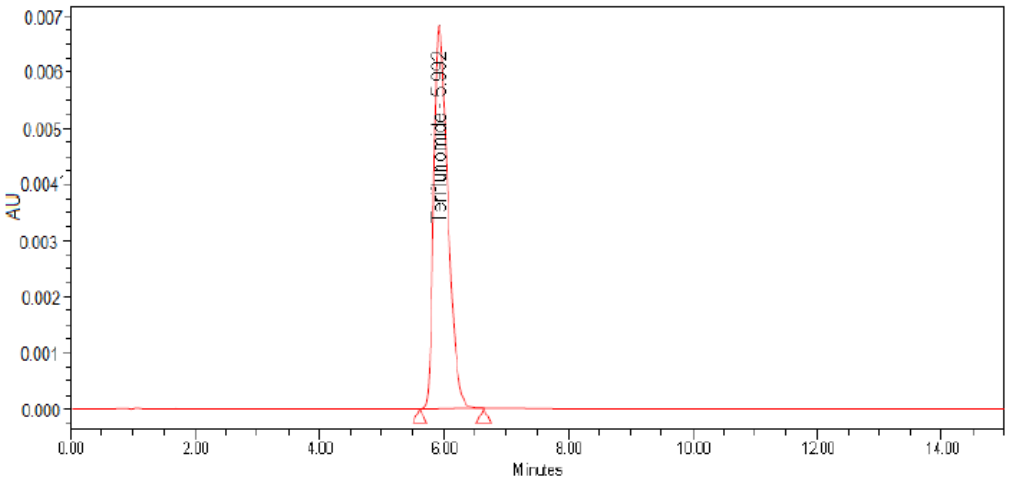
5% Sample 1



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	2	1	5.929	79271	100.00	7696	1.20

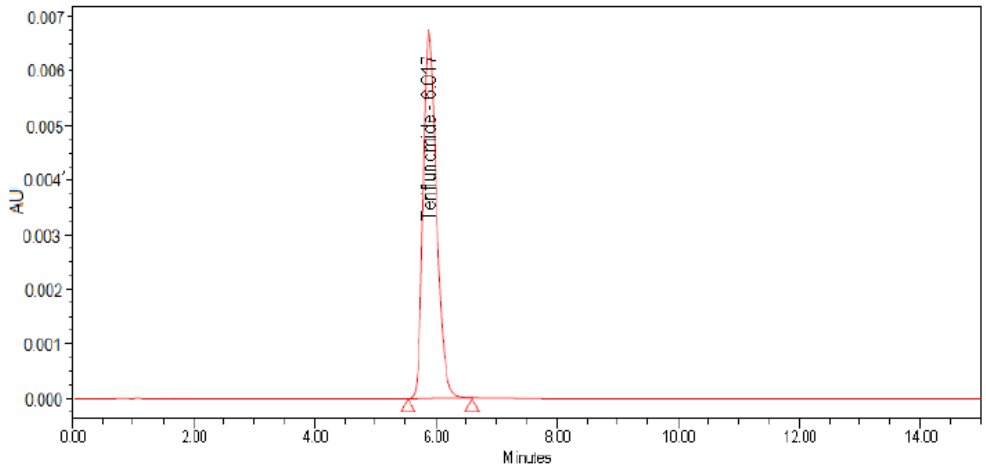
5% Sample 2



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	3	1	5.992	79243	100.00	7622	1.23

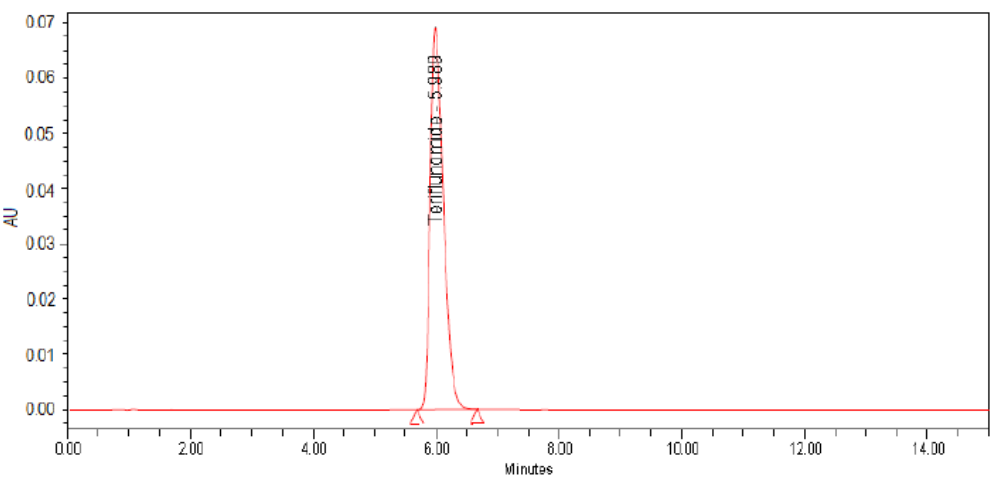
5% Sample 3



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	4	1	6.017	79893	100.00	7741	1.22

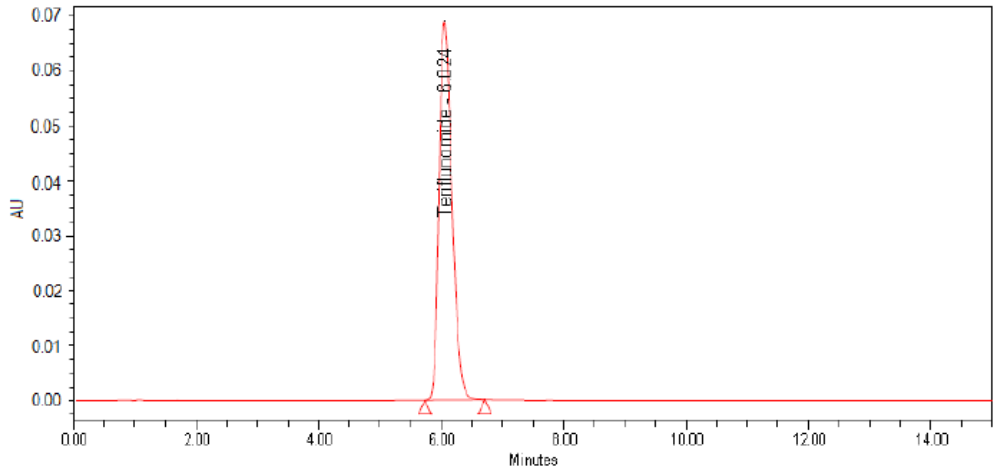
50% Sample 1



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	5	1	5.989	784059	100.00	7685	1.25

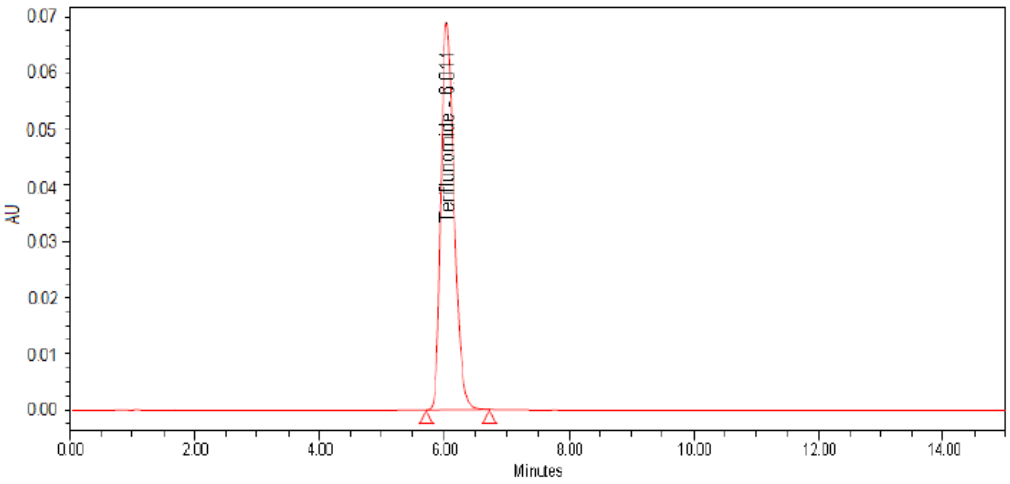
50% Sample 2



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	6	1	6.024	786797	100.00	7652	1.24

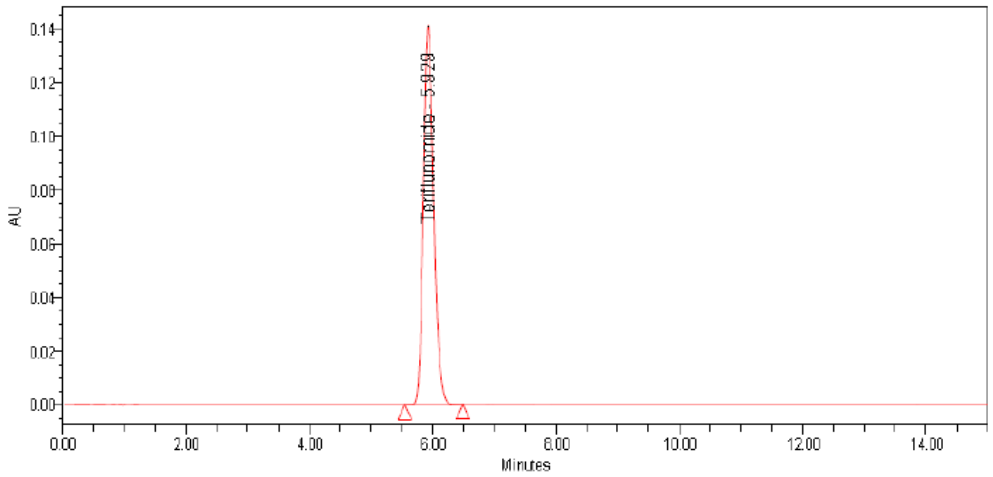
50% Sample 3



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	7	1	6.011	789985	100.00	7602	1.25

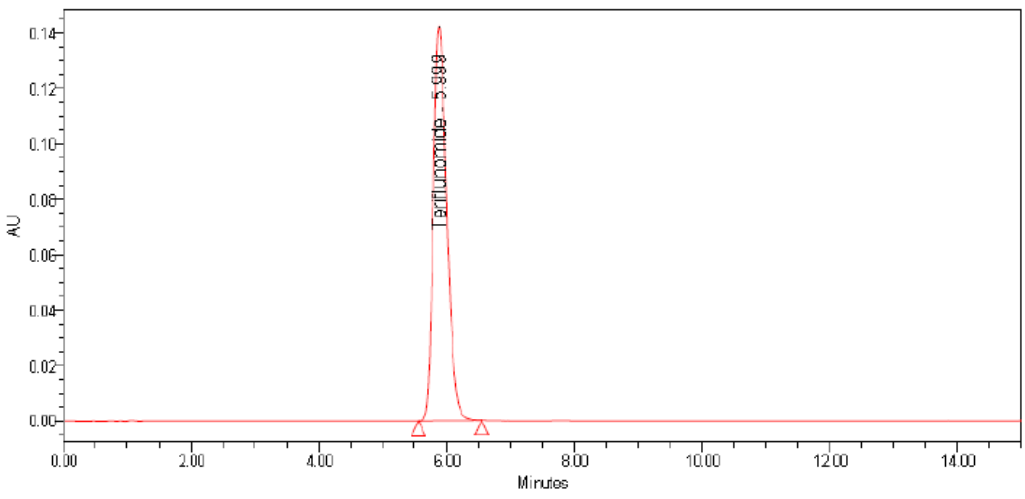
100% Sample 1



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	8	1	5.929	1589177	100.00	7554	1.23

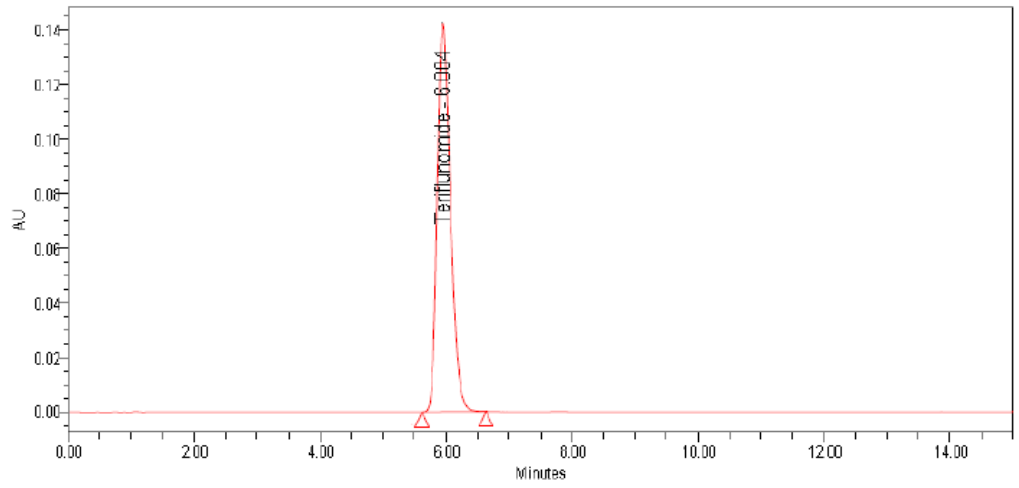
100% Sample 2



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	9	1	5.999	1587887	100.00	7612	1.25

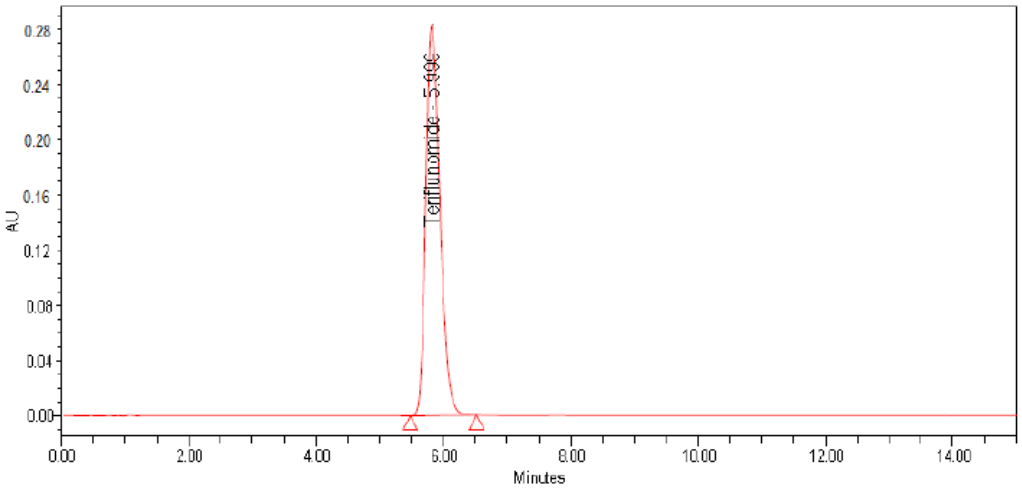
100% Sample 3



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	10	1	6.004	1593464	100.00	7601	1.23

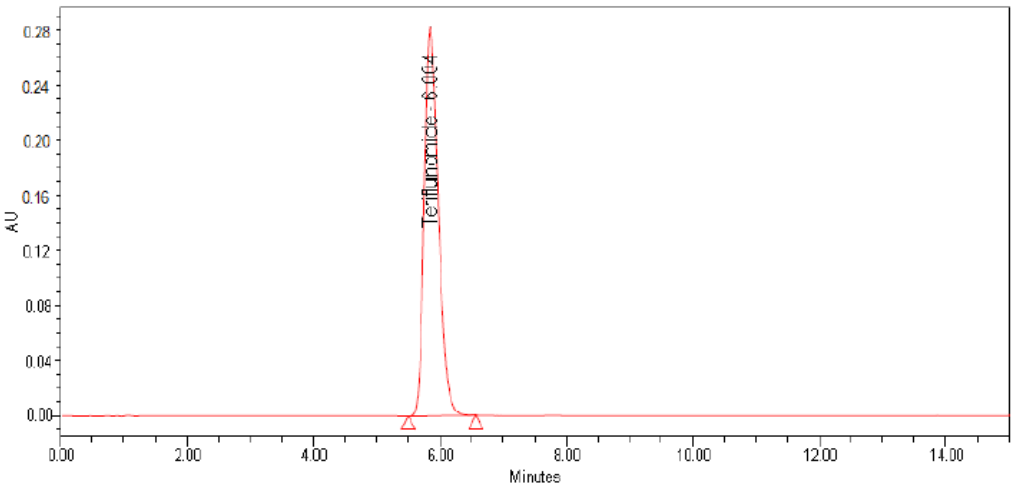
200% Sample 1



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	11	1	5.999	3172132	100.00	7696	1.27

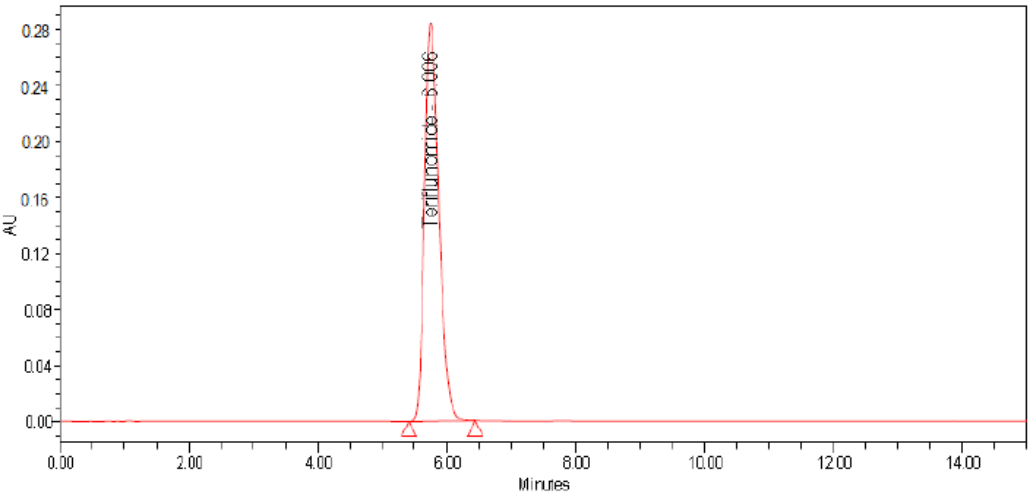
200% Sample 2



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	12	1	6.004	3155725	100.00	7688	1.27

200% Sample 3

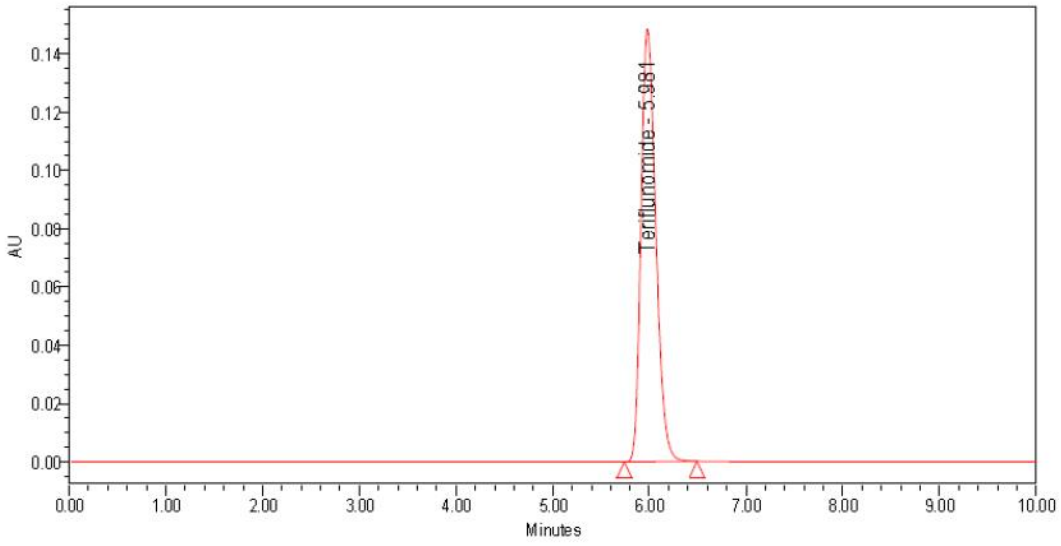


Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	13	1	6.006	3111902	100.00	7532	1.26

Method Precision Chromatograms

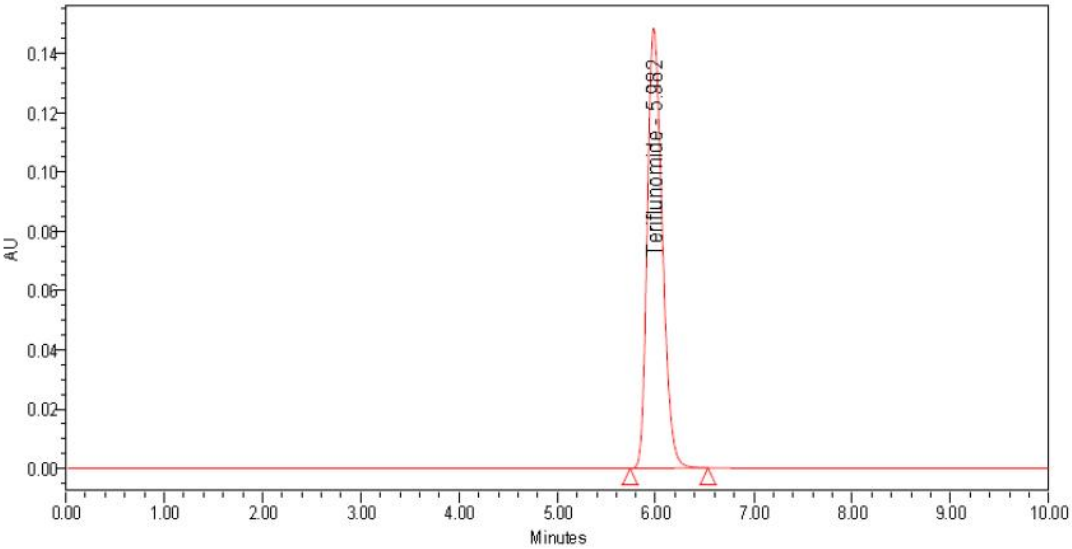
Sample 1 - Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	4	1	5.981	1569920	7241		1.27	BB

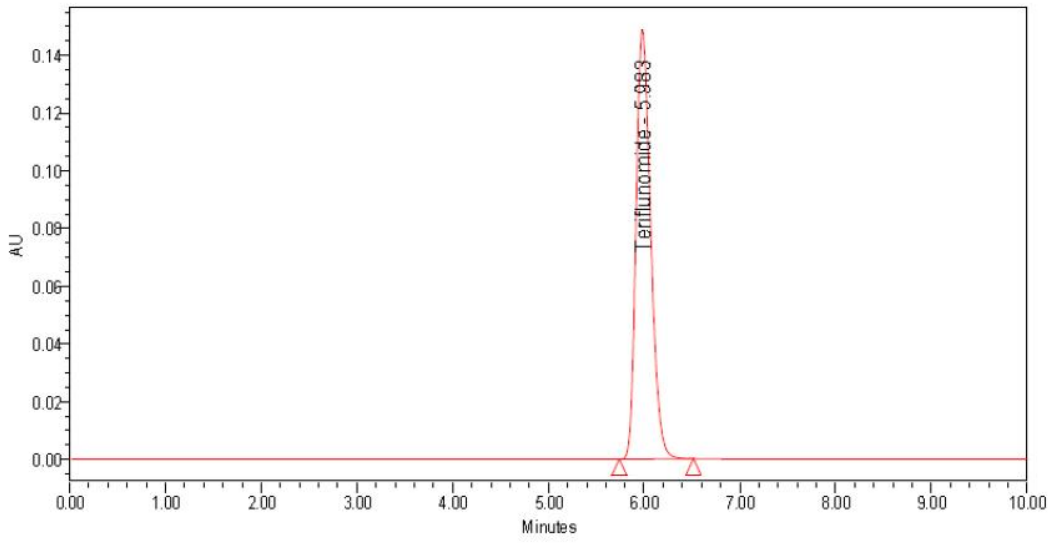
Sample 1 - Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	4	2	5.982	1570709	7244		1.27	BB

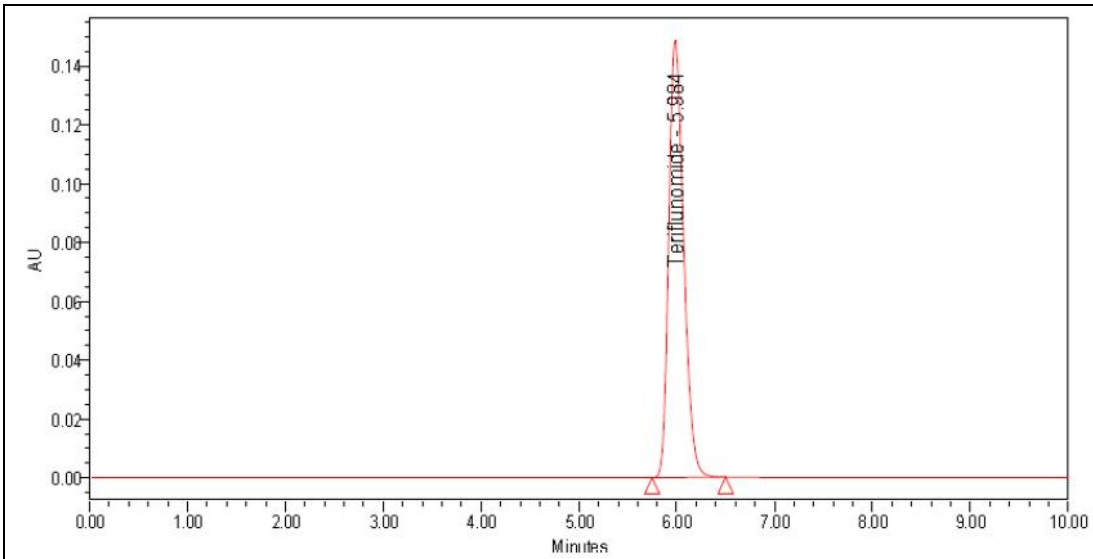
Sample 2 - Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	5	1	5.983	1576413	7224		1.27	BB

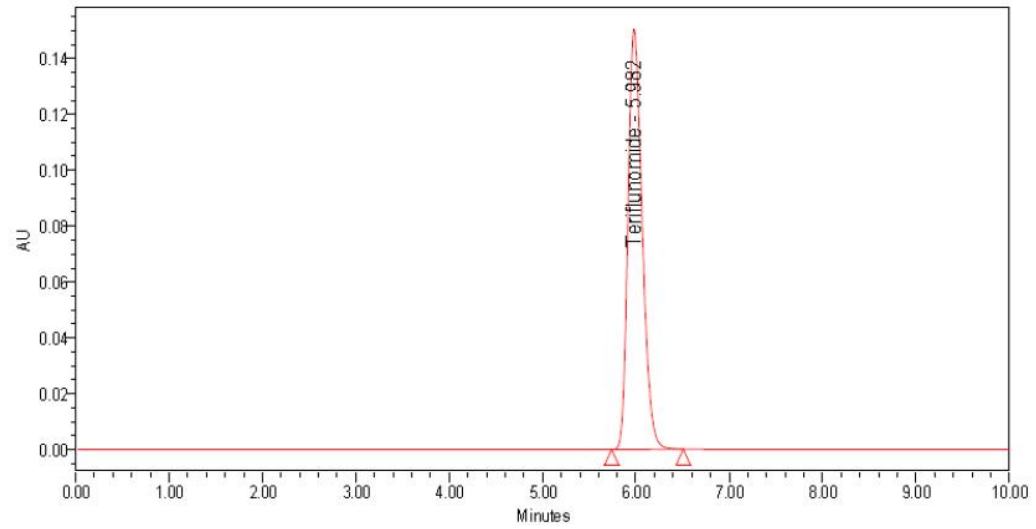
Sample 2 - Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	5	2	5.984	1571605	7253		1.27	BB

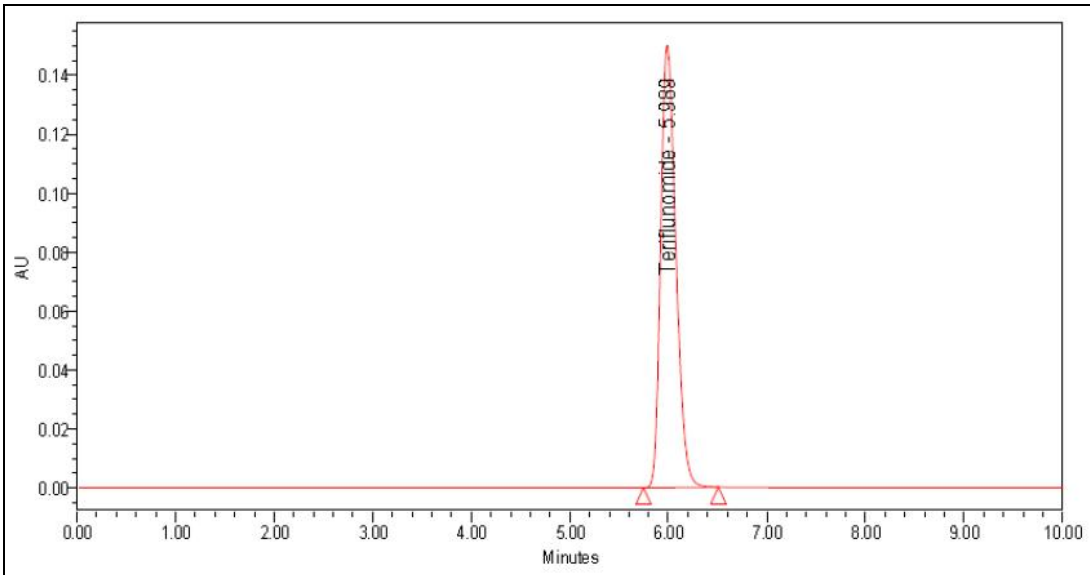
Sample 3 - Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	6	1	5.982	1592749	7233		1.27	BB

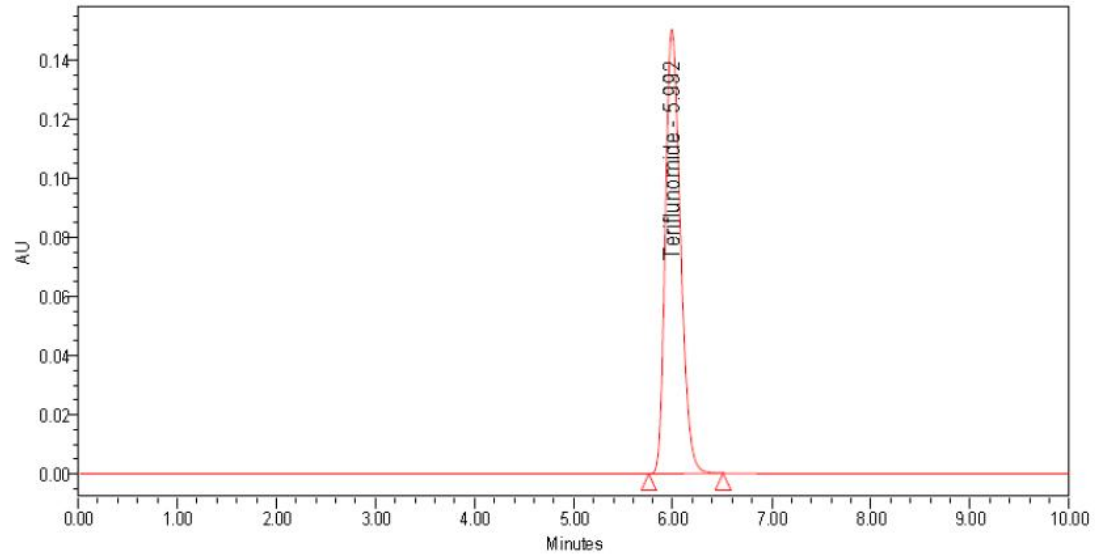
Sample 3 - Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	6	2	5.989	1596950	7176		1.28	BB

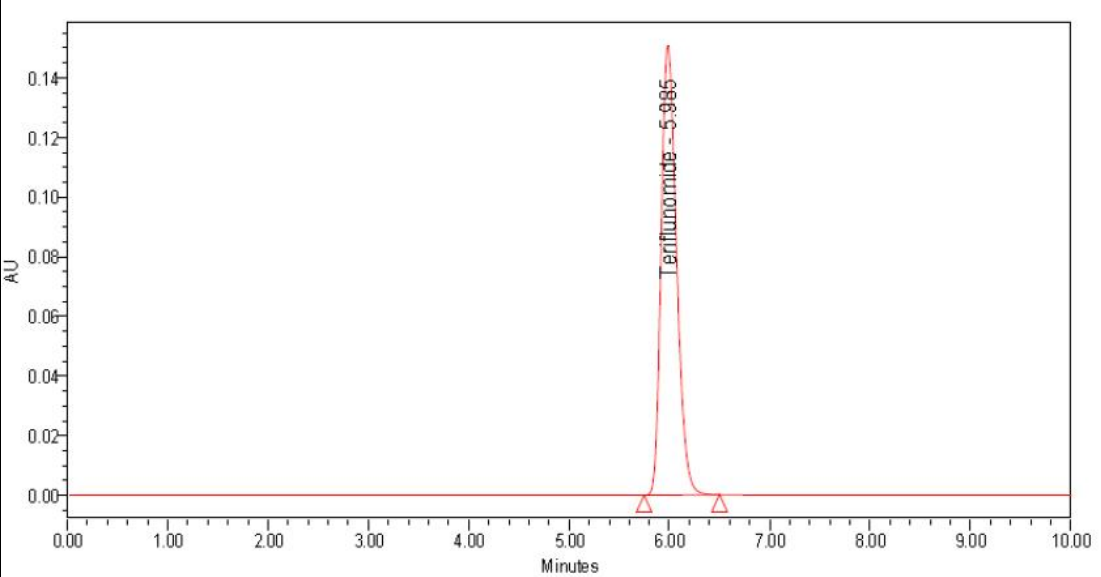
Sample 4 - Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	7	1	5.992	1587115	7281		1.27	BB

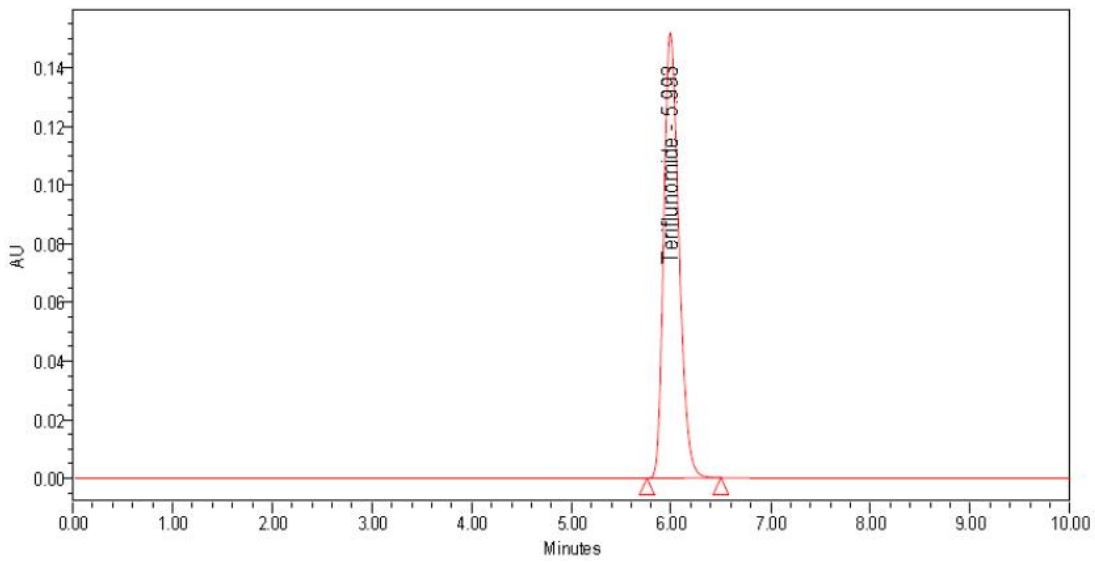
Sample 4 - Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	7	2	5.985	1594383	7247		1.27	BB

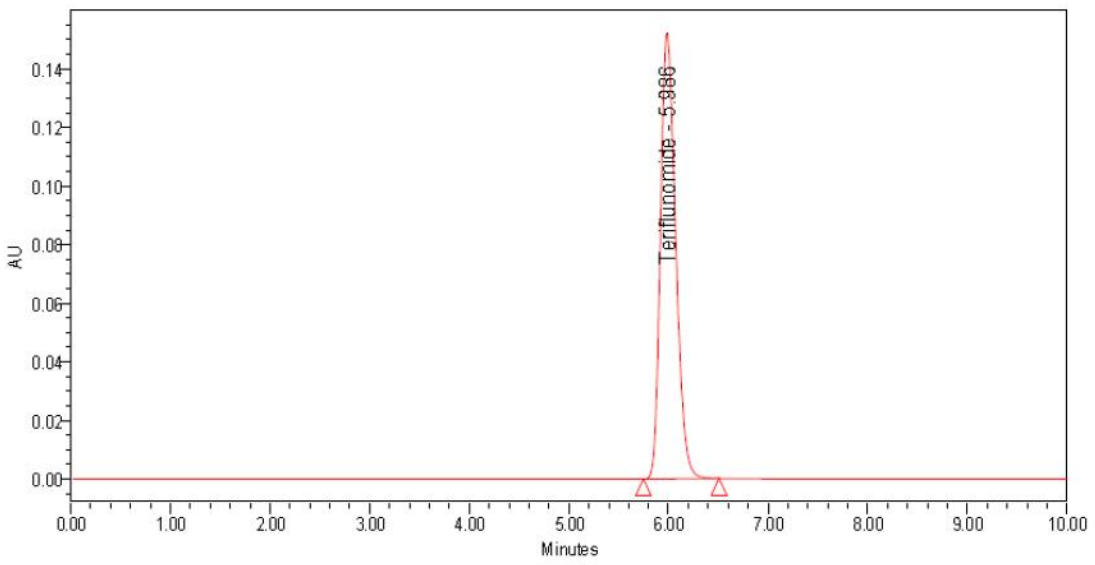
Sample 5 - Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	8	1	5.993	1604535	7280		1.27	BB

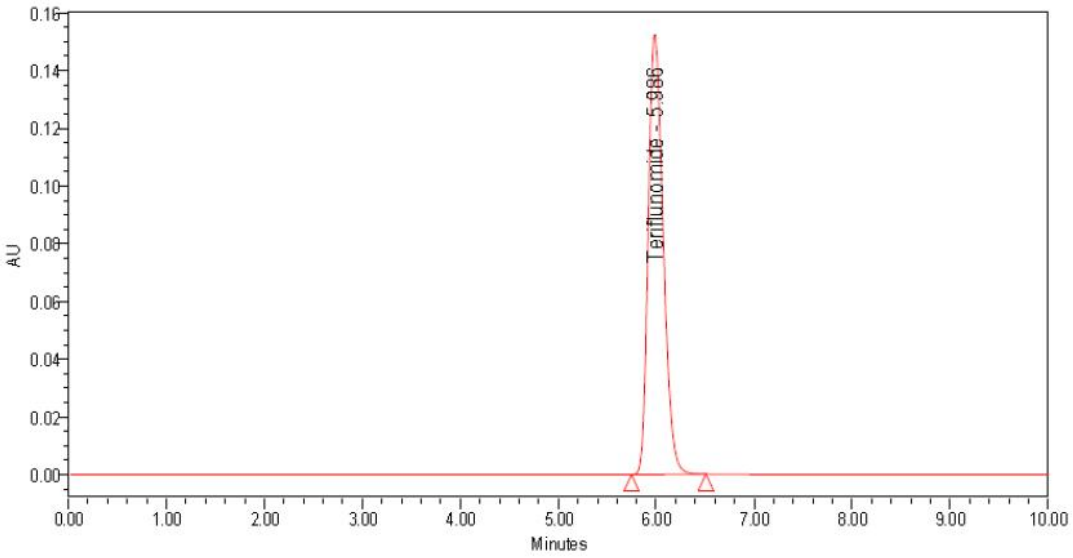
Sample 5 - Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	8	2	5.986	1608421	7250		1.27	BB

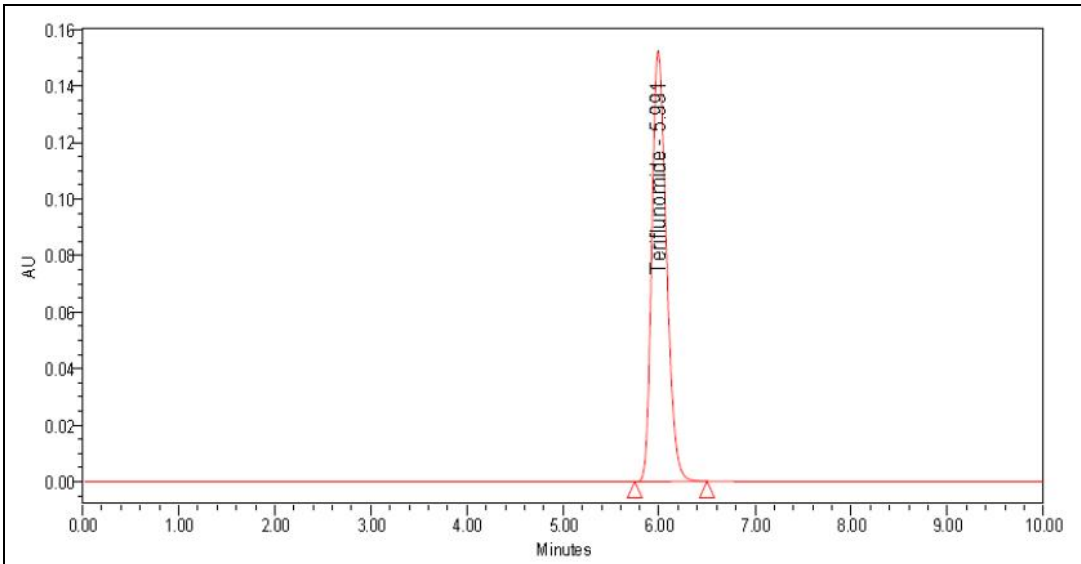
Sample 6 - Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	9	1	5.986	1609650	7255		1.27	BB

Sample 6 - Injection-2

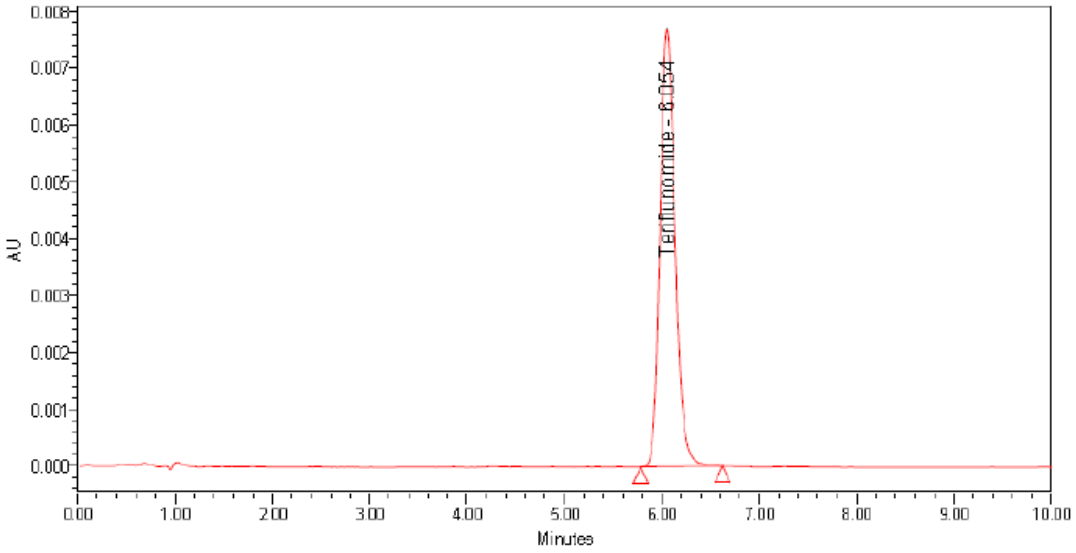


Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	9	2	5.991	1608511	7281		1.27	BB

Linearity Chromatograms

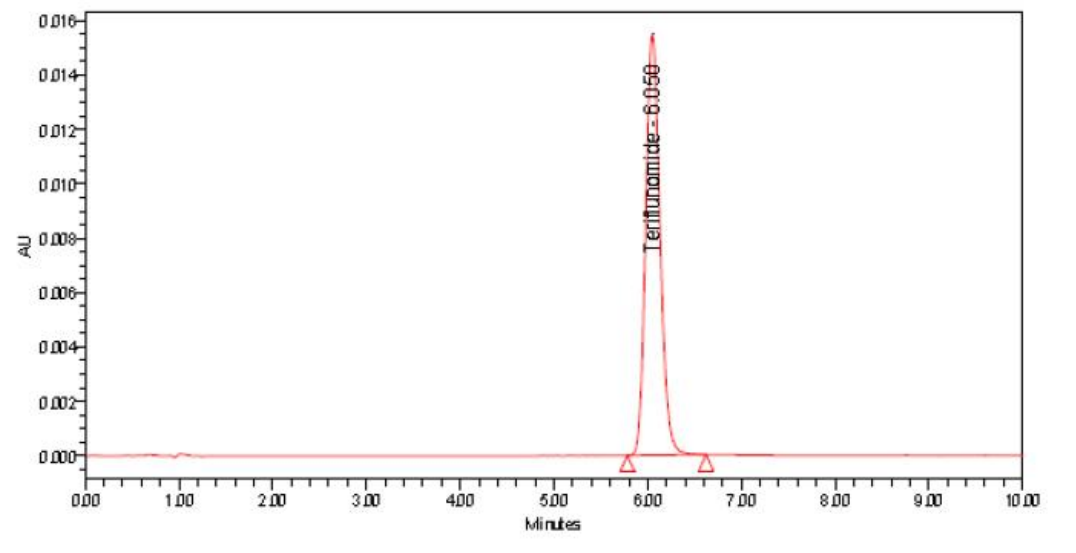
L1 – 5%



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	19	1	6.054	83086	7328		1.17	BB

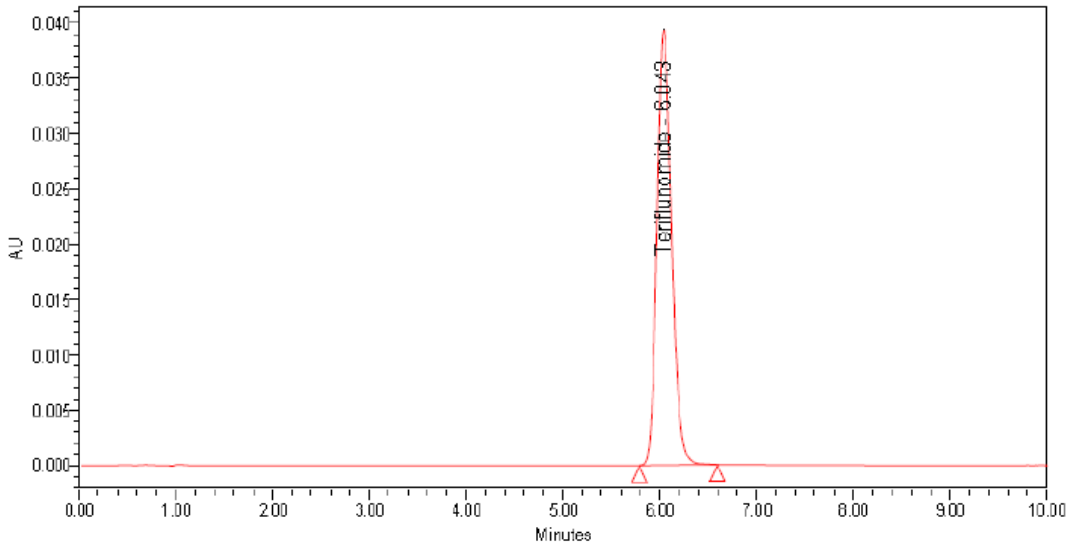
L1 – 10%



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	20	1	6.050	164525	7459		1.17	BB

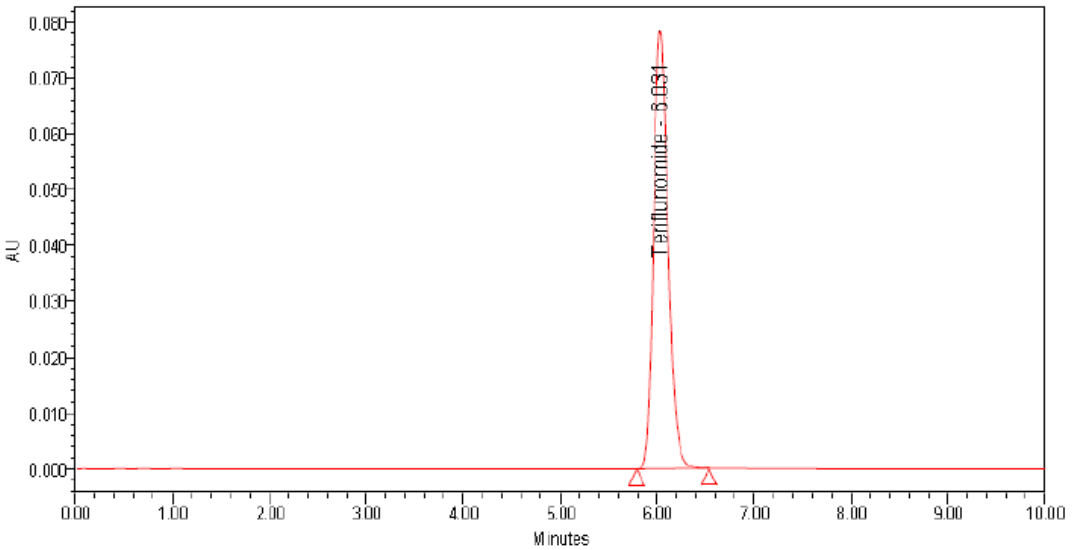
L1 – 25%



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	21	1	6.043	414647	7546		1.18	BB

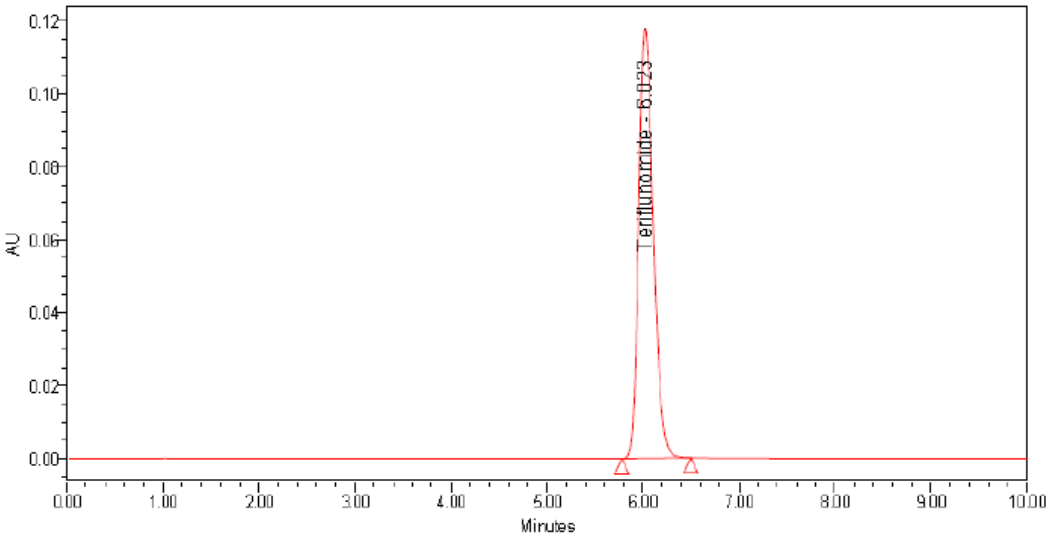
L1 – 50%



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	22	1	6.031	823590	7501		1.22	BB

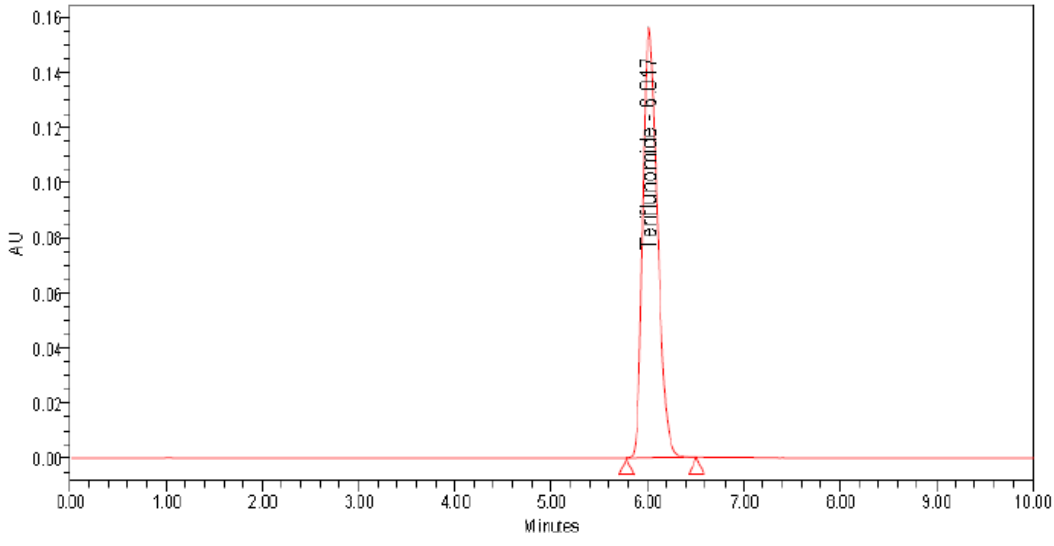
L1 – 75%



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	23	1	6.023	1241003	7420		1.26	BB

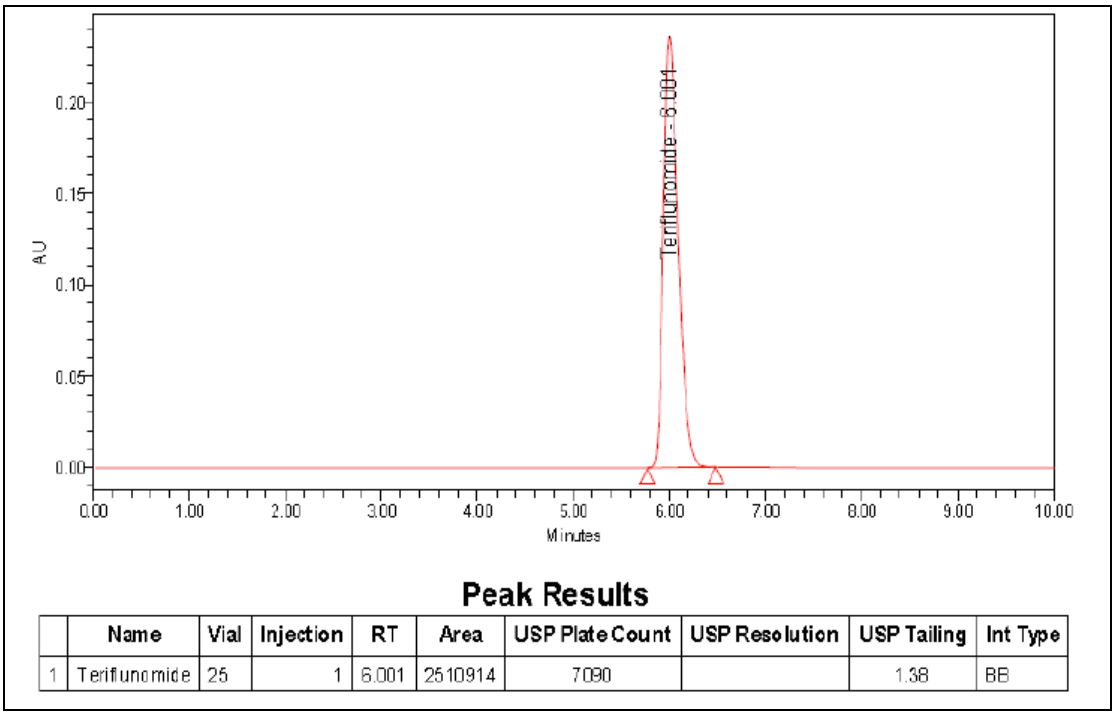
L1 – 100%



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	24	1	6.017	1650624	7337		1.30	BB

L1 – 150%



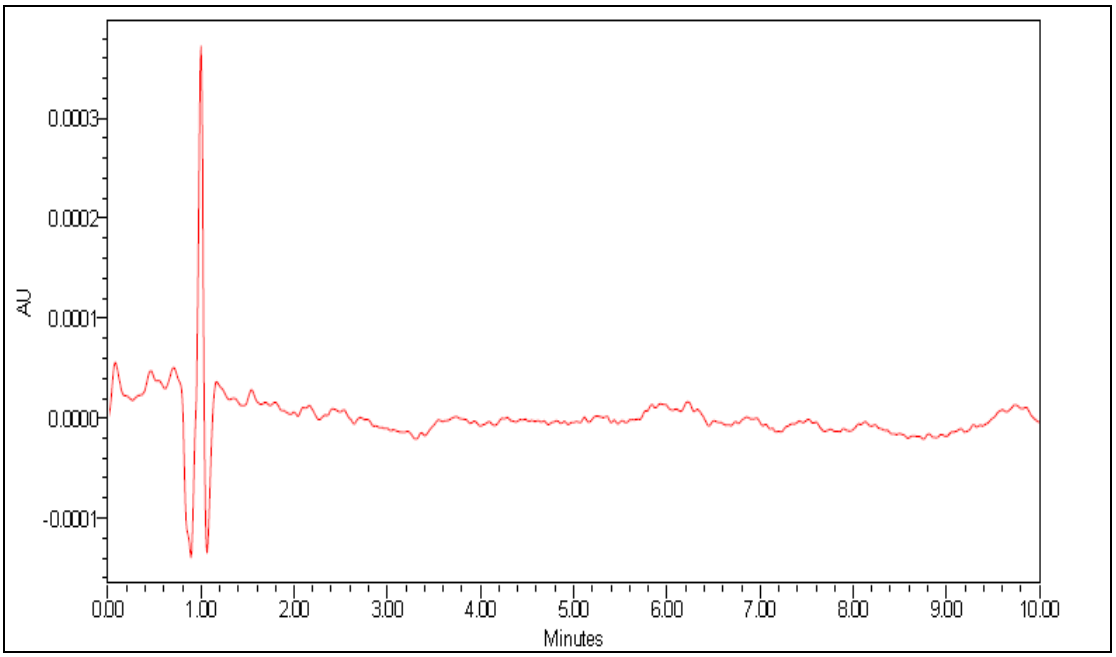
Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	25	1	6.001	2510914	7090		1.38	BB

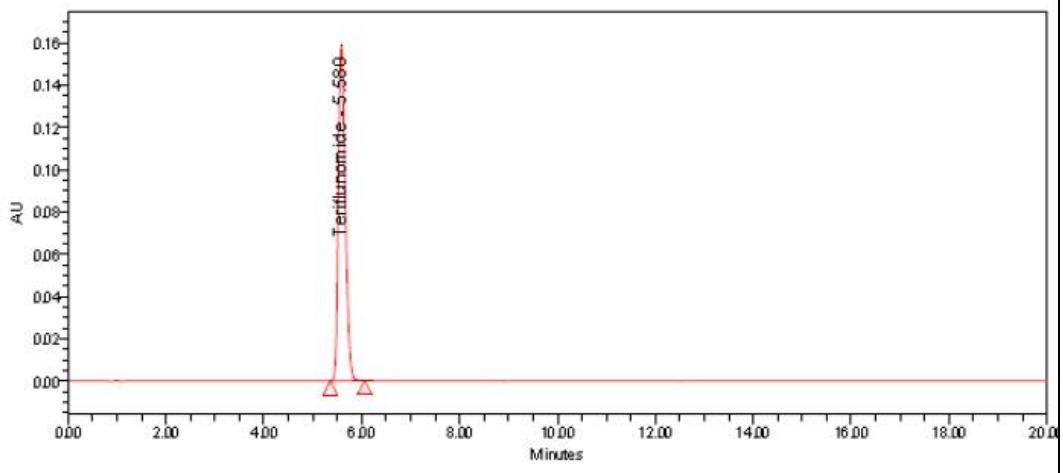
Selectivity/Specificity Chromatograms

Placebo and impurity interference:

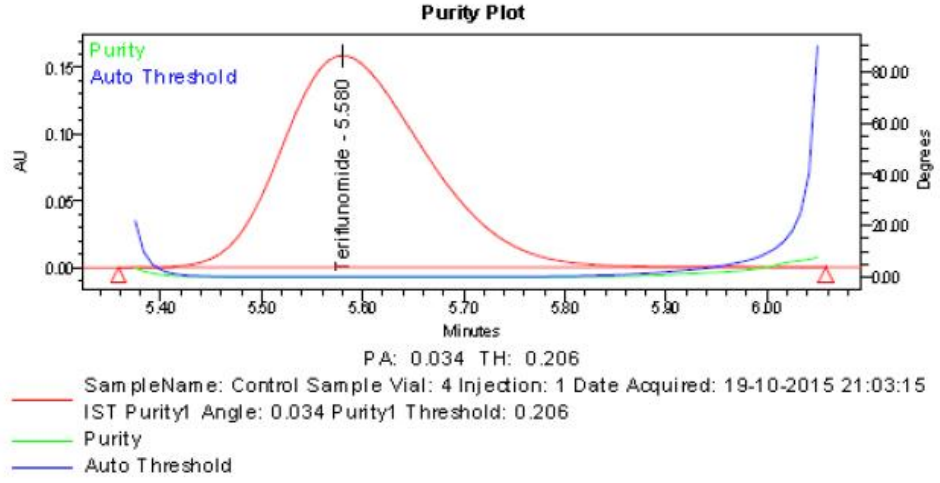
Placebo



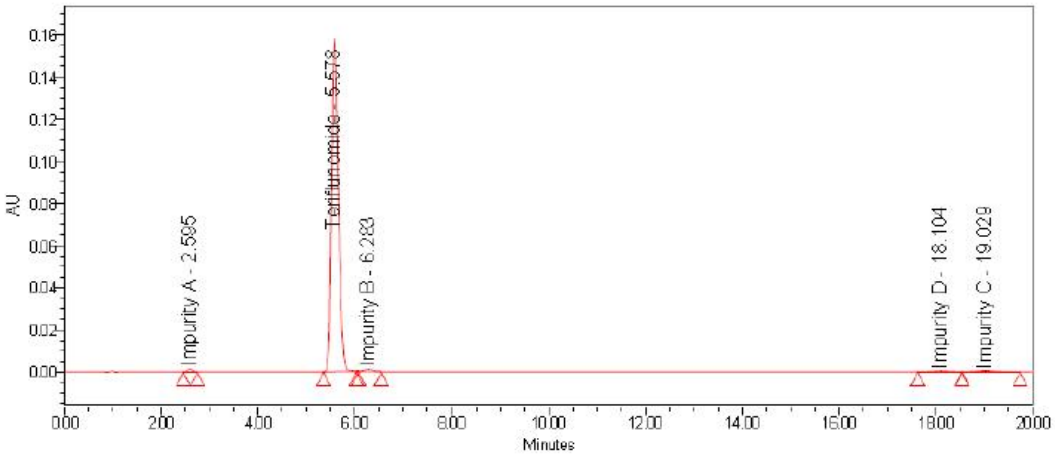
Control Sample



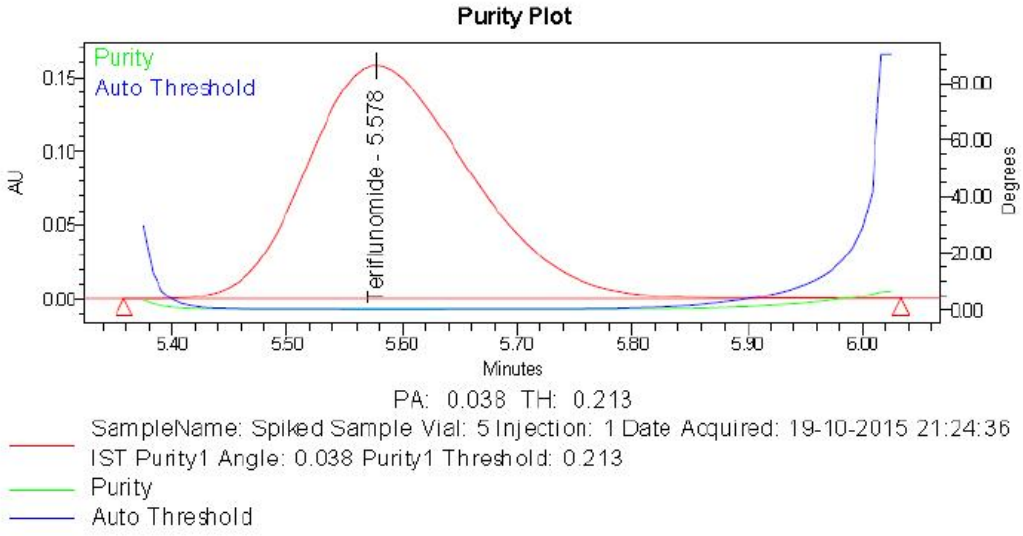
	Name	Vial	Injection	RT	Area (µV*sec)	% Area	Int Type	USP Resolution
1	Impurity A	4	1	2.595			Missing	
2	Teriflunomide	4	1	5.580	1562453	100.00	BB	
3	Impurity B	4	1	6.282			Missing	
4	Impurity D	4	1	18.107			Missing	
5	Impurity C	4	1	19.027			Missing	



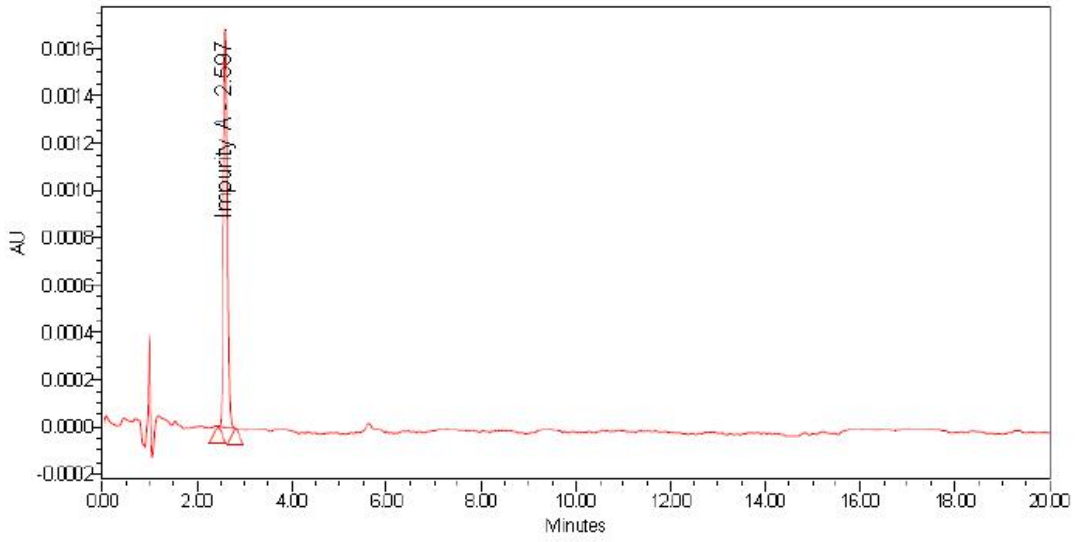
Spiked Sample



	Name	Vial	Injection	RT	Area (µV*sec)	% Area	Int Type	USP Resolution
1	Impurity A	5	1	2.595	9755	0.61	BB	
2	Teriflunomide	5	1	5.578	1556410	97.34	BB	14.08
3	Impurity B	5	1	6.283	11208	0.70	BB	2.58
4	Impurity D	5	1	18.104	9467	0.59	BB	24.91
5	Impurity C	5	1	19.029	12173	0.76	BB	1.34



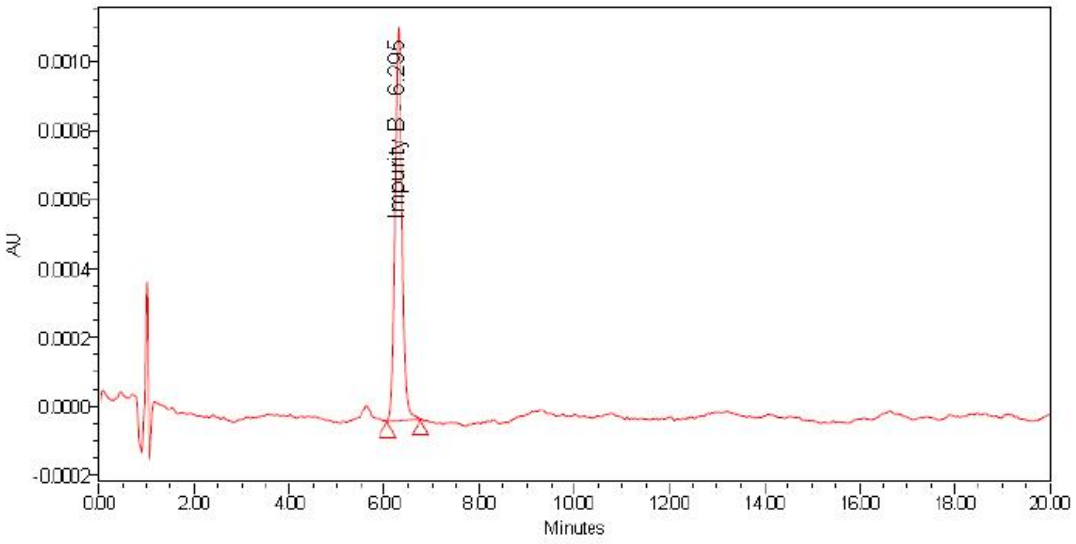
Impurity A



Peak Results

	Name	Vial	Injection	RT	Int Type	Area	% Area
1	Impurity A	7	1	2.597	BB	10021	100.00
2	Teriflunomide	7	1	5.580	Missing		
3	Impurity B	7	1	6.282	Missing		
4	Impurity D	7	1	18.107	Missing		
5	Impurity C	7	1	19.027	Missing		

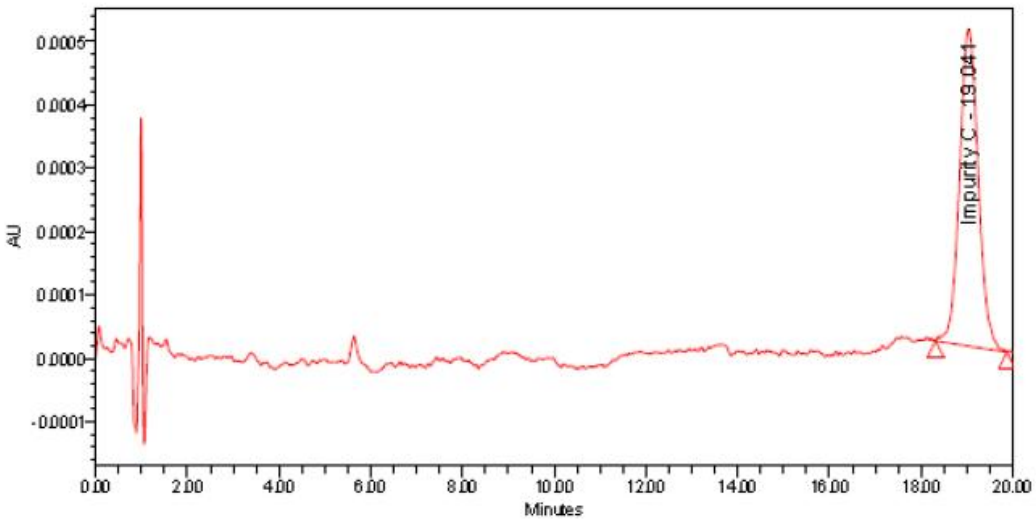
Impurity B



Peak Results

	Name	Vial	Injection	RT	Int Type	Area	% Area
1	Impurity A	8	1	2.595	Missing		
2	Teriflunomide	8	1	5.580	Missing		
3	Impurity B	8	1	6.295	BB	12289	100.00
4	Impurity D	8	1	18.107	Missing		
5	Impurity C	8	1	19.027	Missing		

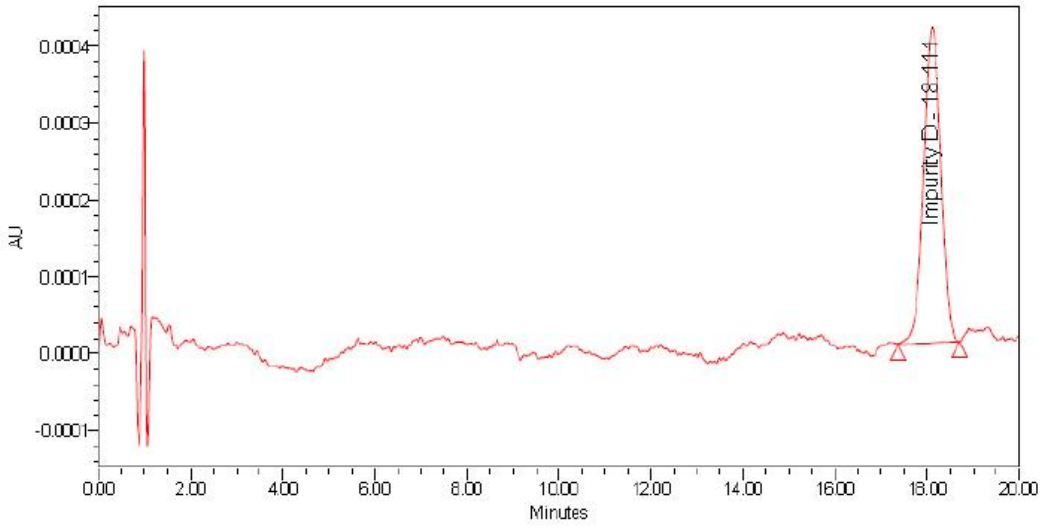
Impurity C



Peak Results

	Name	Vial	Injection	RT	Int Type	Area	% Area
1	Impurity A	9	1	2.595	Missing		
2	Teriflunomide	9	1	5.580	Missing		
3	Impurity B	9	1	6.282	Missing		
4	Impurity D	9	1	18.107	Missing		
5	Impurity C	9	1	19.041	BB	14121	100.00

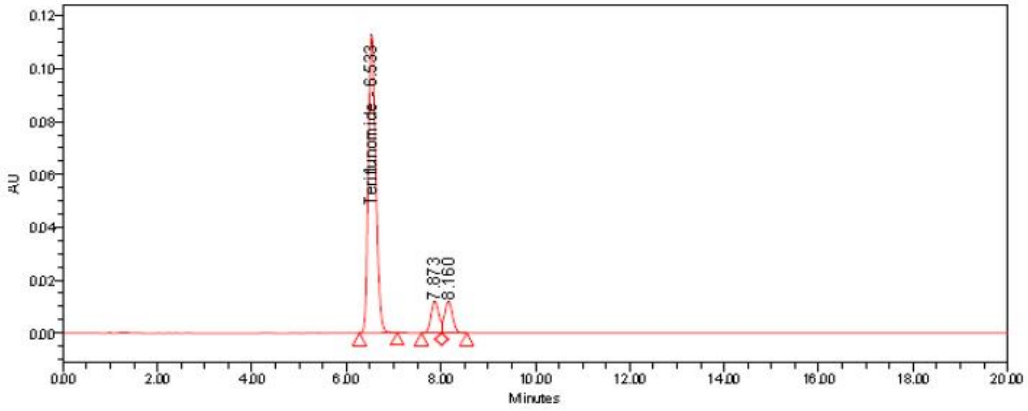
Impurity D



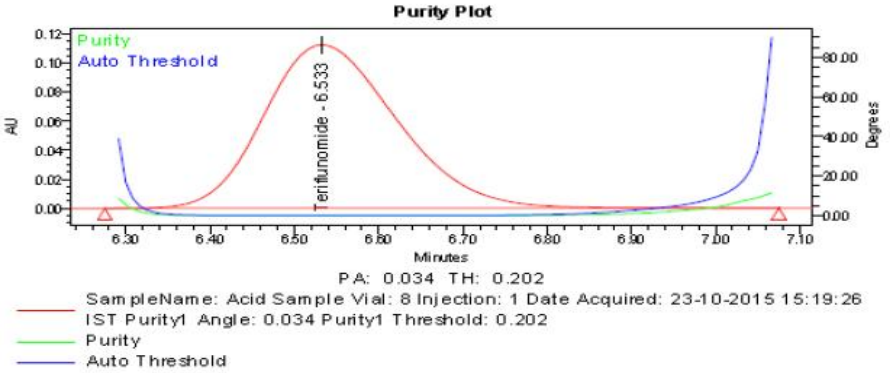
Peak Results

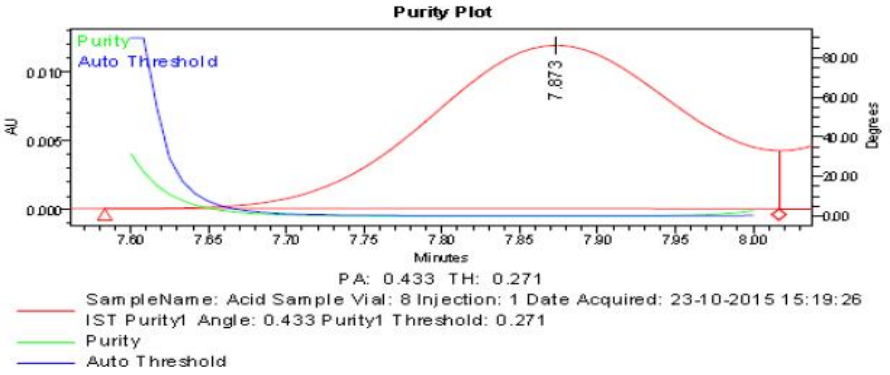
	Name	Vial	Injection	RT	Int Type	Area	% Area
1	Impurity A	10	1	2.595	Missing		
2	Teriflunomide	10	1	5.580	Missing		
3	Impurity B	10	1	6.282	Missing		
4	Impurity D	10	1	18.111	BB	10690	100.00
5	Impurity C	10	1	19.027	Missing		

Interference from degradation products:
Acid stress

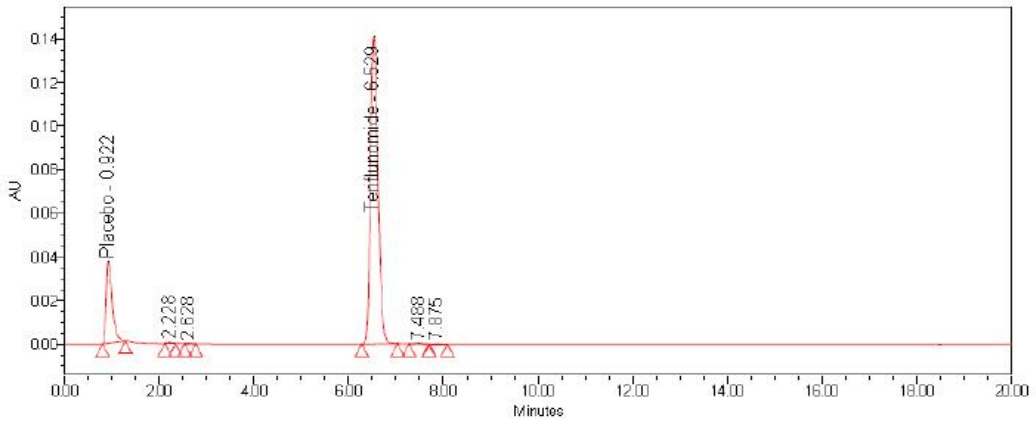


	Name	Vial	Injection	RT	Area (µV*sec)	% Area	Int Type	USP Resolution
1	Placebo	8	1	0.900			Missing	
2	Peroxide	8	1	0.950			Missing	
3	Impurity A	8	1	2.595			Missing	
4	Teriflunomide	8	1	6.533	1235008	81.77	BB	
5	Impurity B	8	1	6.700			Missing	
6		8	1	7.873	135072	8.94	BV	4.36
7		8	1	8.160	140289	9.29	VB	0.88
8	Impurity D	8	1	20.000			Missing	
9	Impurity C	8	1	21.000			Missing	

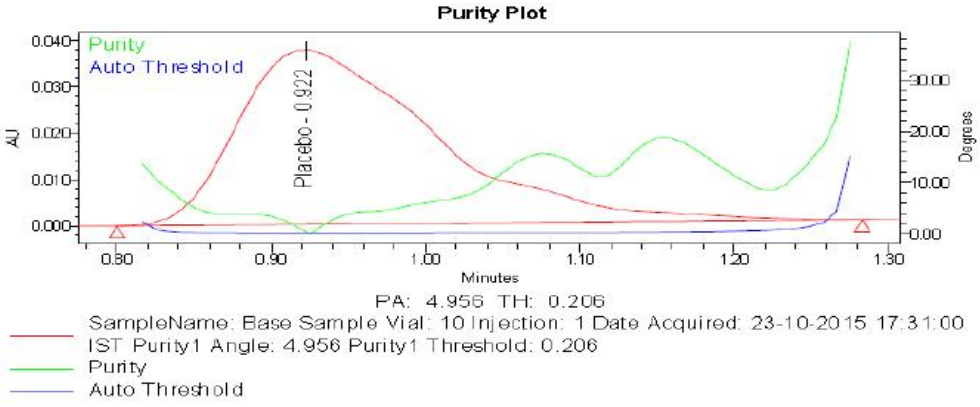


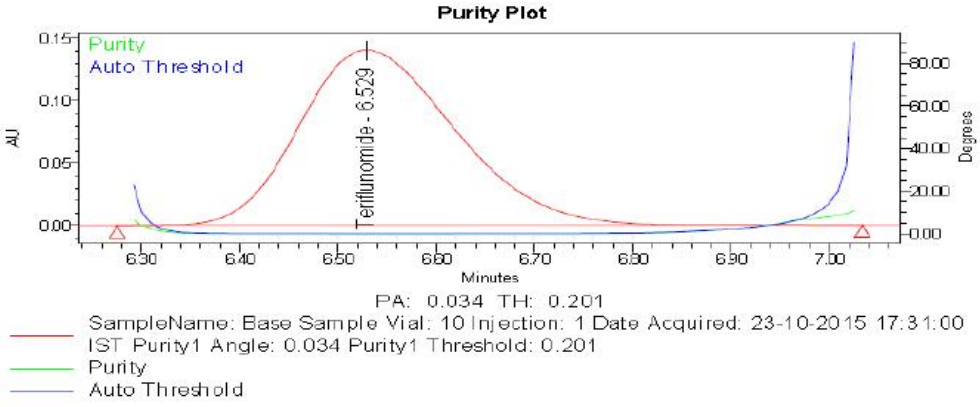


Base stress

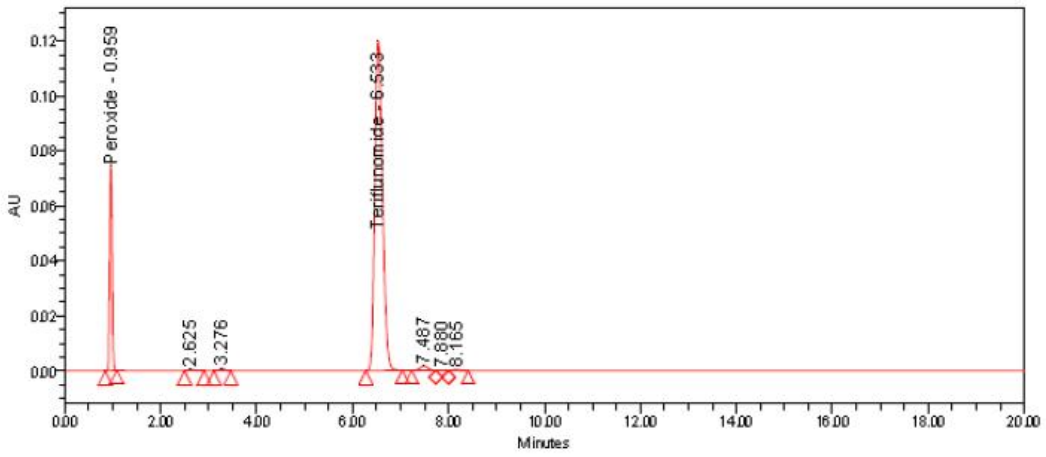


	Name	Vial	Injection	RT	Area (µV*sec)	% Area	Int Type	USP Resolution
1	Placebo	10	1	0.922	333842	17.61	BB	
2	Peroxide	10	1	0.950			Missing	
3		10	1	2.228	2886	0.15	BB	6.70
4	Impurity A	10	1	2.595			Missing	
5		10	1	2.628	1245	0.07	BB	2.43
6	Teriflunomide	10	1	6.529	1554163	81.97	BB	16.92
7	Impurity B	10	1	6.700			Missing	
8		10	1	7.488	2840	0.15	BB	3.30
9		10	1	7.875	1010	0.05	BB	1.36
10	Impurity D	10	1	20.000			Missing	
11	Impurity C	10	1	21.000			Missing	



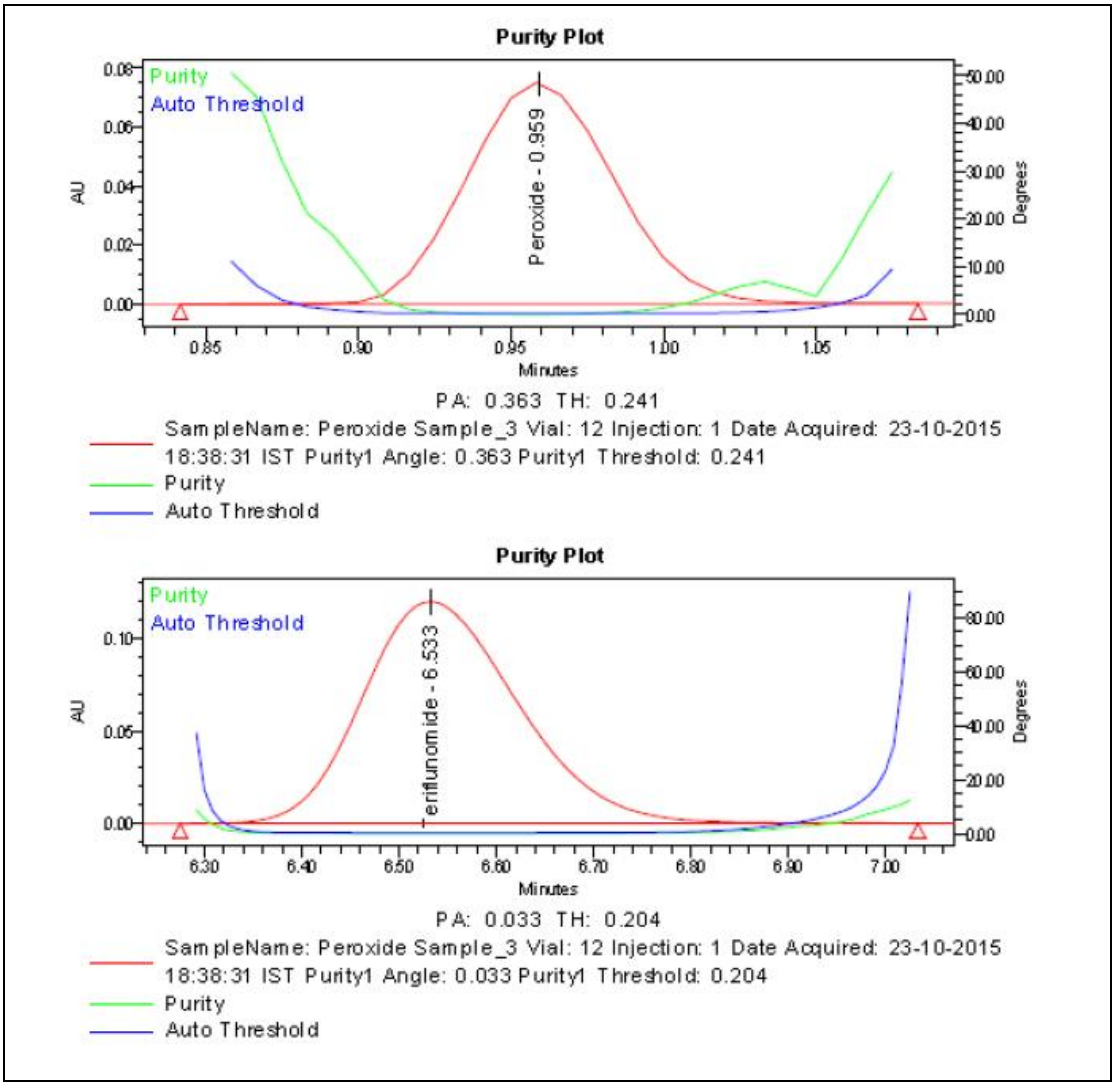


Peroxide stress

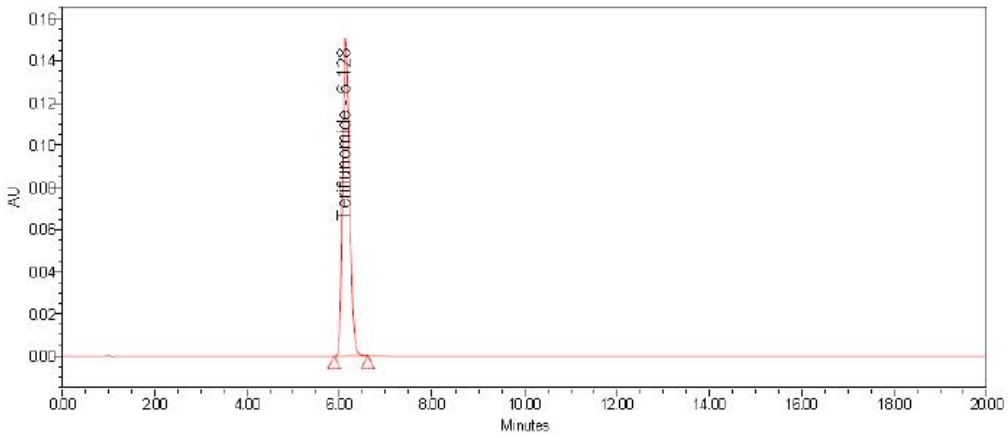


	Name	Vial	Injection	RT	Area (µV*sec)	% Area	Int Type	USP Resolution
1	Placebo	12	1	0.900			Missing	
2	Peroxide	12	1	0.959	254011	15.73	BB	
3	Impurity A	12	1	2.595			Missing	
4		12	1	2.625	4154	0.26	BB	11.62
5		12	1	3.276	6405	0.40	BB	3.59
6	Teriflunomide	12	1	6.533	1326435	82.15	BB	13.98
7	Impurity B	12	1	6.700			Missing	
8		12	1	7.487	18344	1.14	BV	3.21
9		12	1	7.880	1645	0.10	VV	1.30
10		12	1	8.165	3706	0.23	VB	0.92
11	Impurity D	12	1	20.000			Missing	

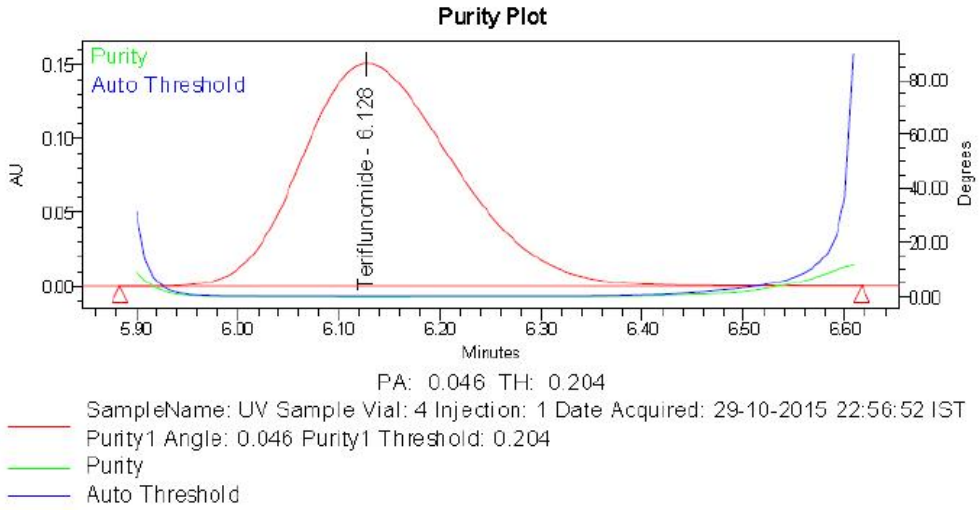
	Name	Vial	Injection	RT	Area (µV*sec)	% Area	Int Type	USP Resolution
12	Impurity C	12	1	21.000			Missing	


Purity Plot
Purity
Auto Threshold
Peroxide - 0.959
AU
Degrees
Minutes
PA: 0.363 TH: 0.241
SampleName: Peroxide Sample_3 Vial: 12 Injection: 1 Date Acquired: 23-10-2015 18:38:31 IST Purity1 Angle: 0.363 Purity1 Threshold: 0.241
Purity
Auto Threshold
Purity Plot
Purity
Auto Threshold
eriflunomide - 6.533
AU
Degrees
Minutes
PA: 0.033 TH: 0.204
SampleName: Peroxide Sample_3 Vial: 12 Injection: 1 Date Acquired: 23-10-2015 18:38:31 IST Purity1 Angle: 0.033 Purity1 Threshold: 0.204
Purity
Auto Threshold

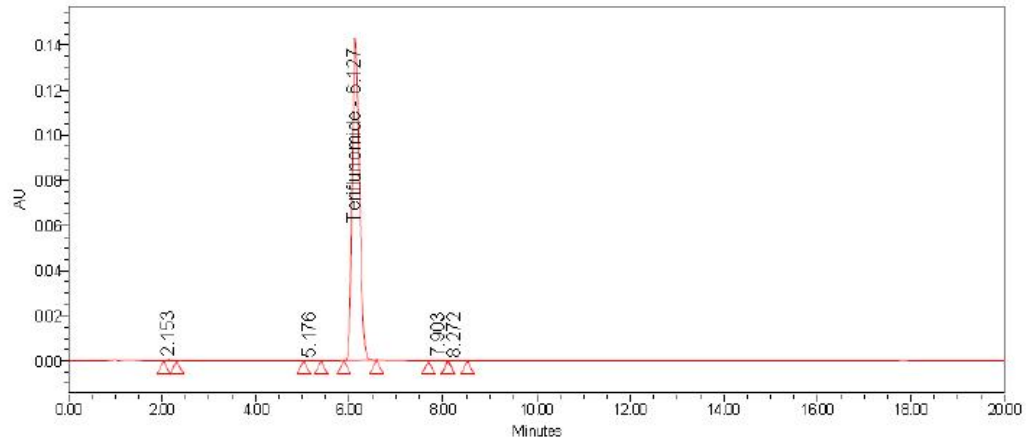
UV light stress



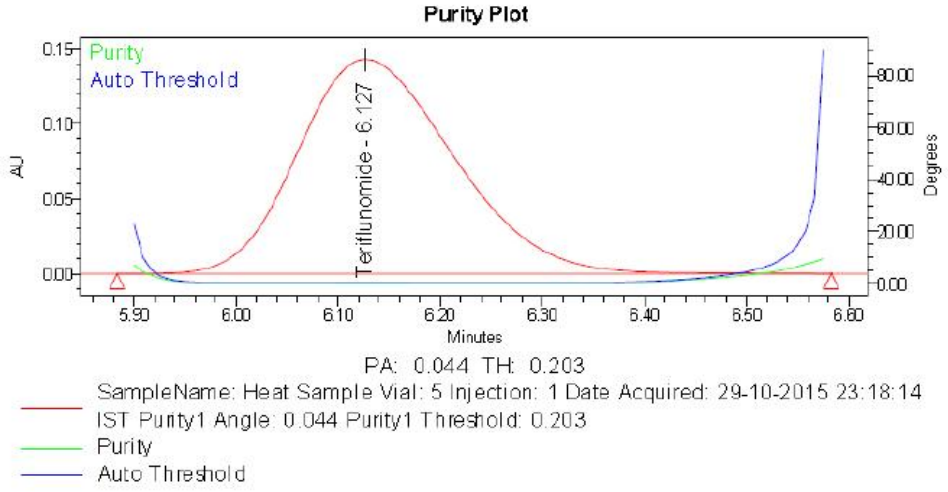
	Name	Vial	Injection	RT	Area (µV*sec)	% Area	Int Type	USP Resolution
1	Teriflunomide	4	1	6.128	1581102	100.00	BB	



Heat stress

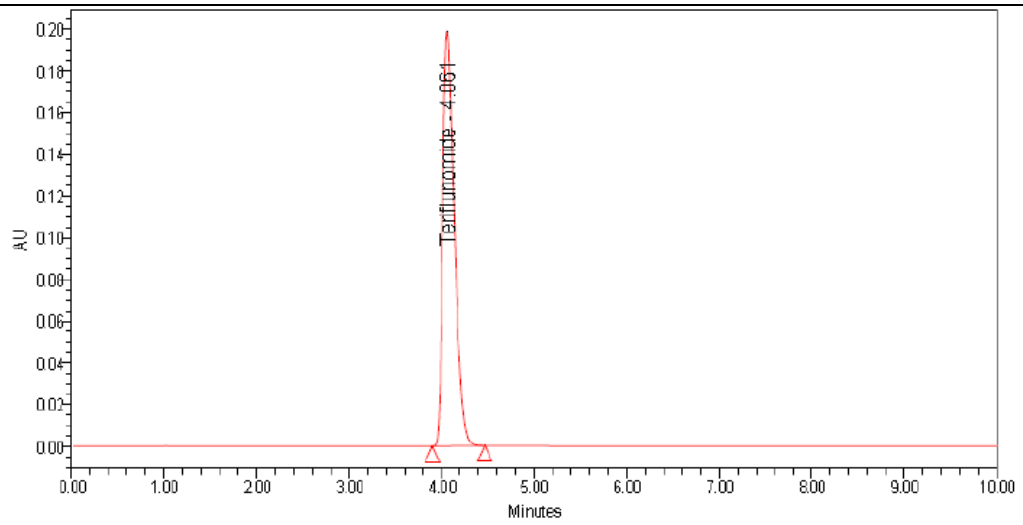


	Name	Vial	Injection	RT	Area (µV*sec)	% Area	Int Type	USP Resolution
1		5	1	2.153	2053	0.14	BB	
2		5	1	5.176	713	0.05	BB	15.56
3	Teriflunomide	5	1	6.127	1515478	99.68	BB	3.79
4		5	1	7.903	1295	0.09	BB	6.09
5		5	1	8.272	871	0.06	BB	1.23



Solution Stability

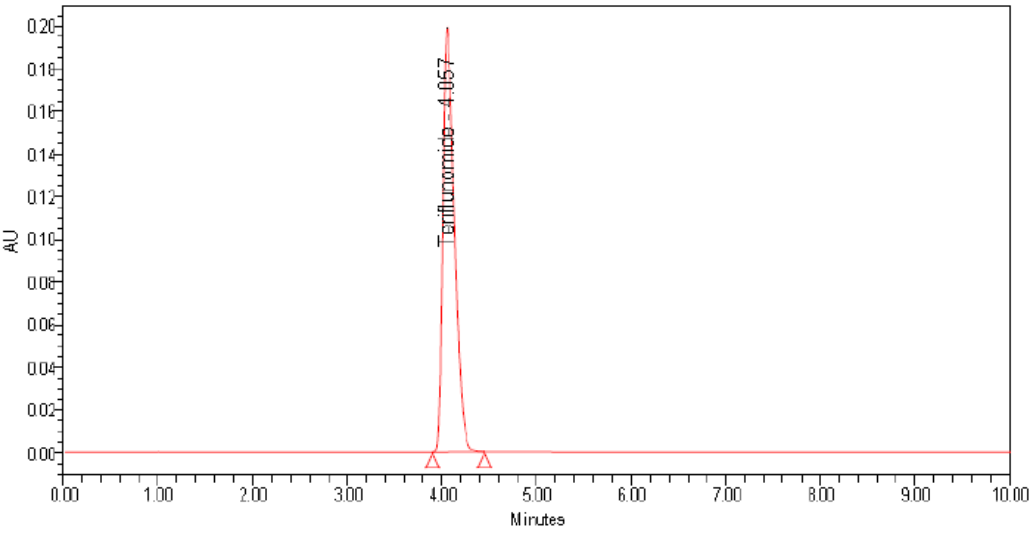
Standard – 24 Hrs



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	2	1	4.061	1624042	100.00	7513	1.35

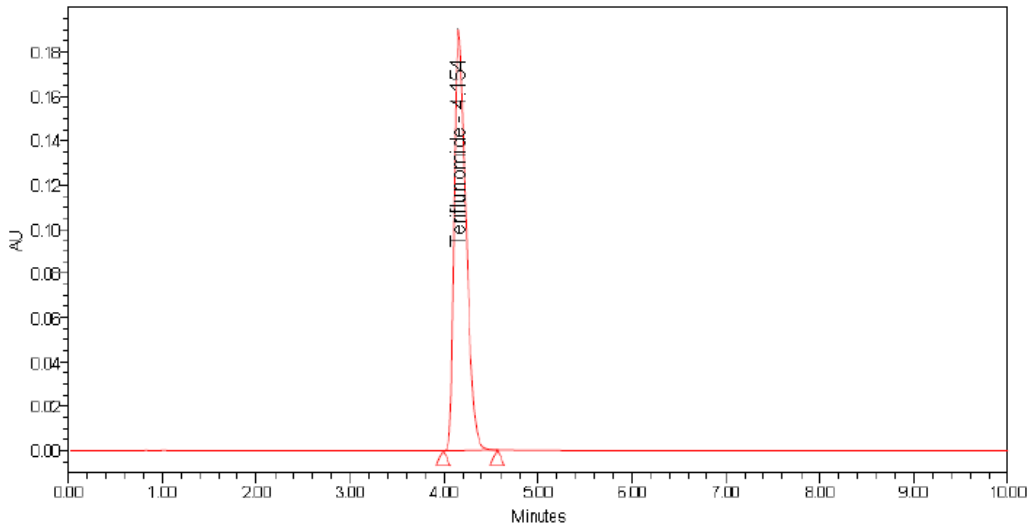
Standard – 48 Hrs



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	3	1	4.057	1633427	100.00	7593	1.32

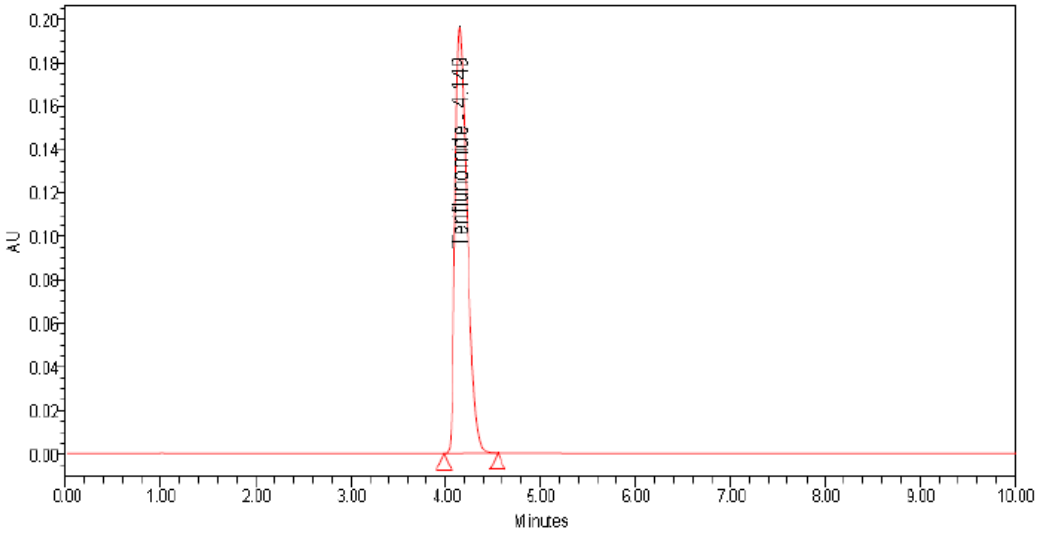
Sample – 24 Hrs



Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	3	1	4.154	1582351	100.00	7502	1.33

Sample – 48 Hrs

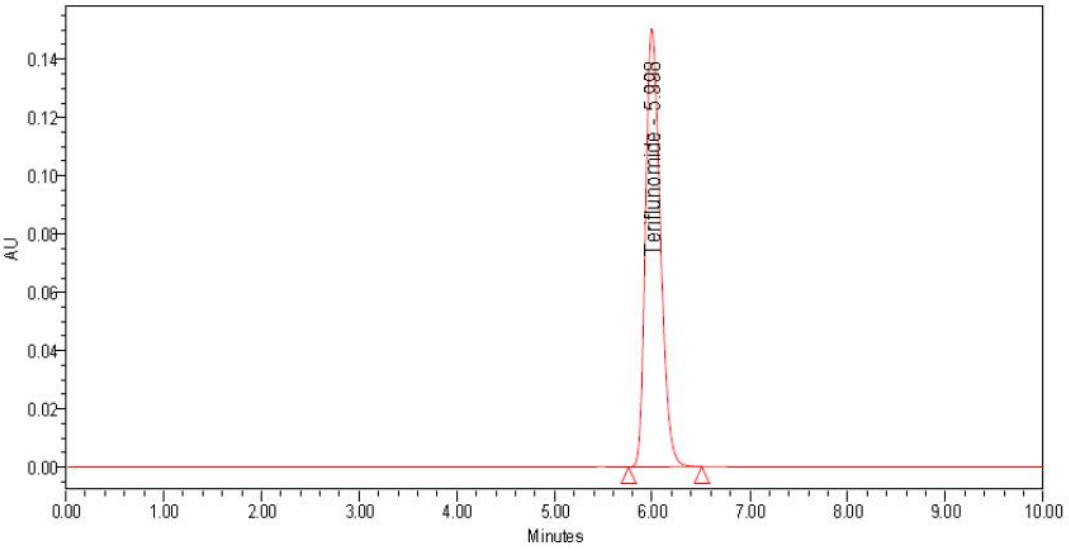


Peak Results

	Name	Vial	Injection	RT	Area (µV*sec)	% Area	USP Plate count	USP Tailing
	Teriflunomide	5	1	4.149	1583676	100.00	7458	1.37

Filter Study

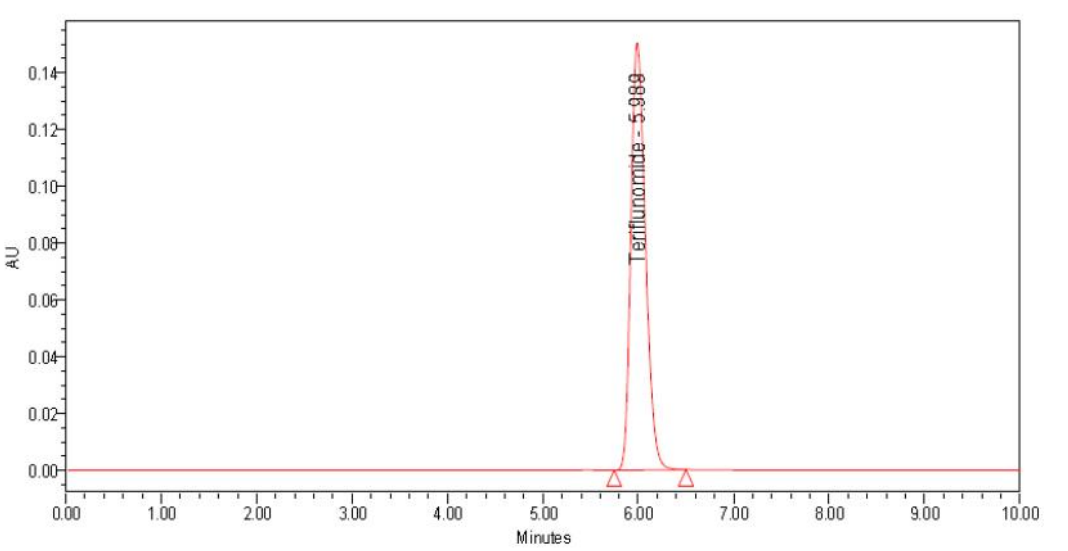
Centrifuged Sample (10 min @ 3500rpm)
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	10	1	5.998	1585790	7317		1.27	BB

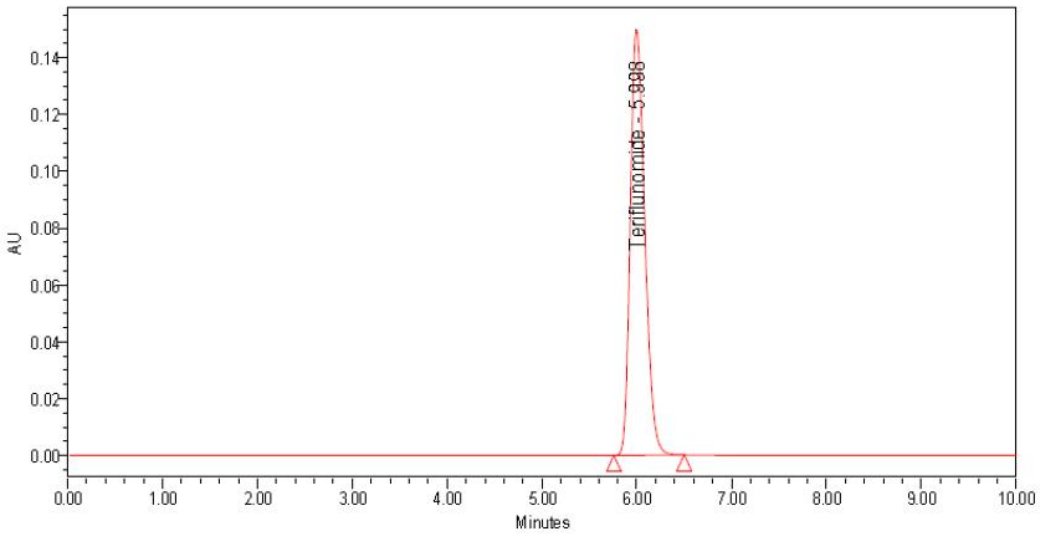
Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	10	2	5.989	1586268	7289		1.27	BB

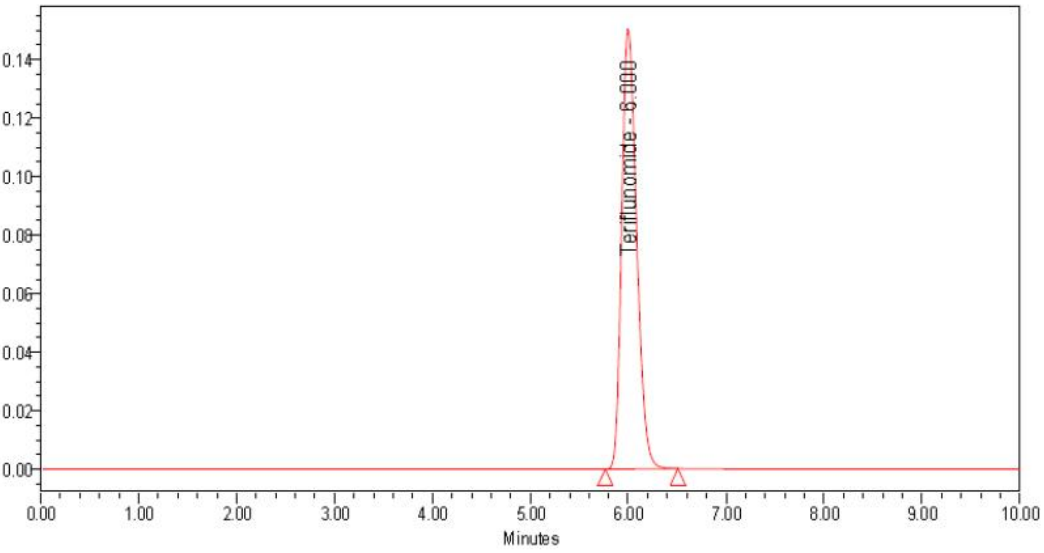
0.45µ PVDF filtrate sample, 3 mL discarded
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	11	1	5.998	1577216	7335		1.27	BB

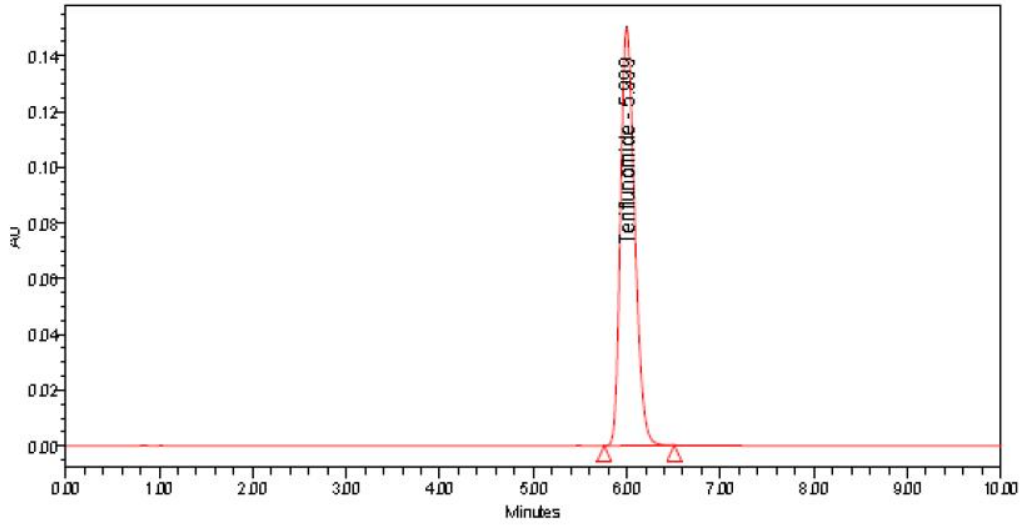
Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	11	2	6.000	1583485	7337		1.27	BB

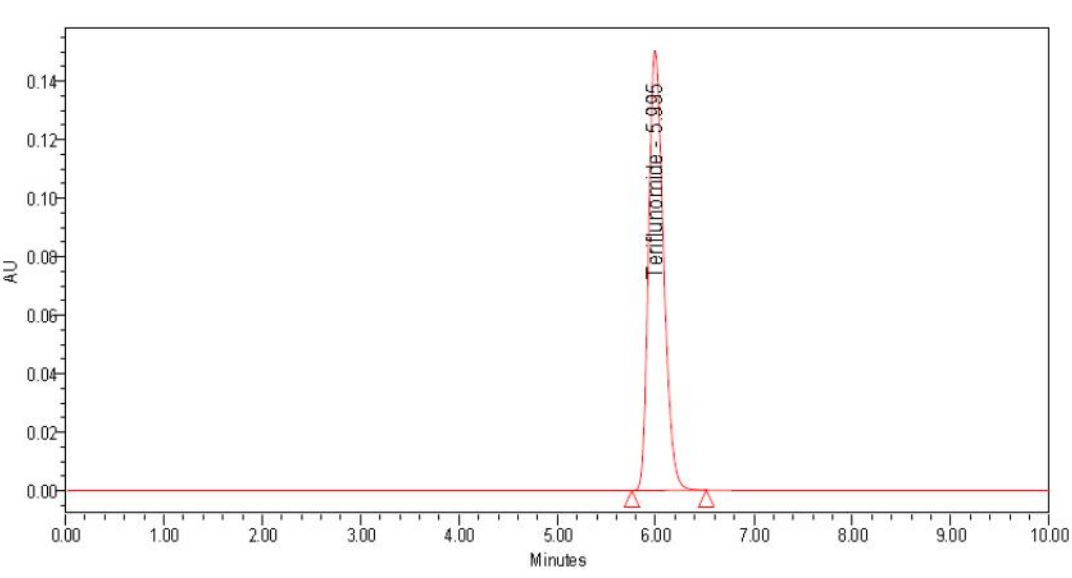
0.45µ PVDF filtrate sample, 4 mL discarded
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	12	1	5.999	1582821	7328		1.27	BB

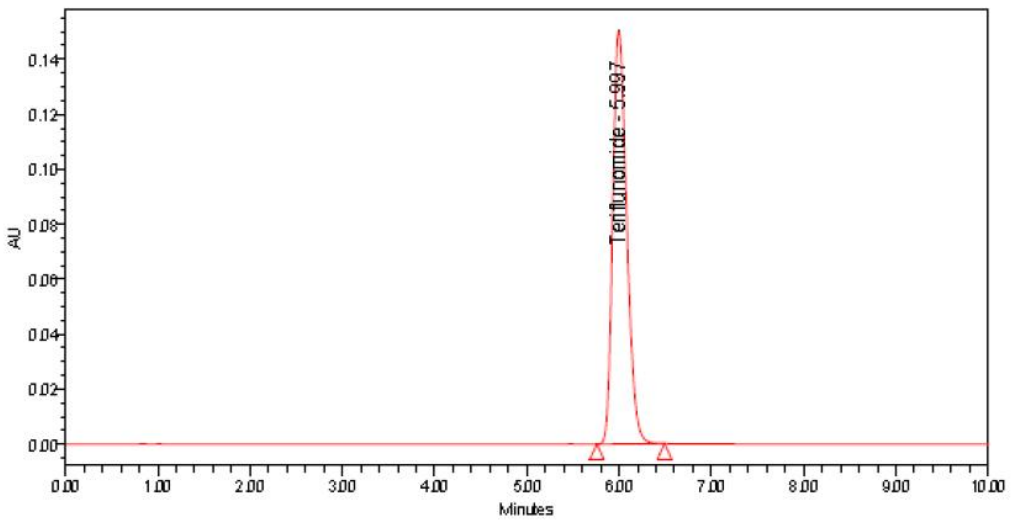
Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	12	2	5.995	1582444	7330		1.28	BB

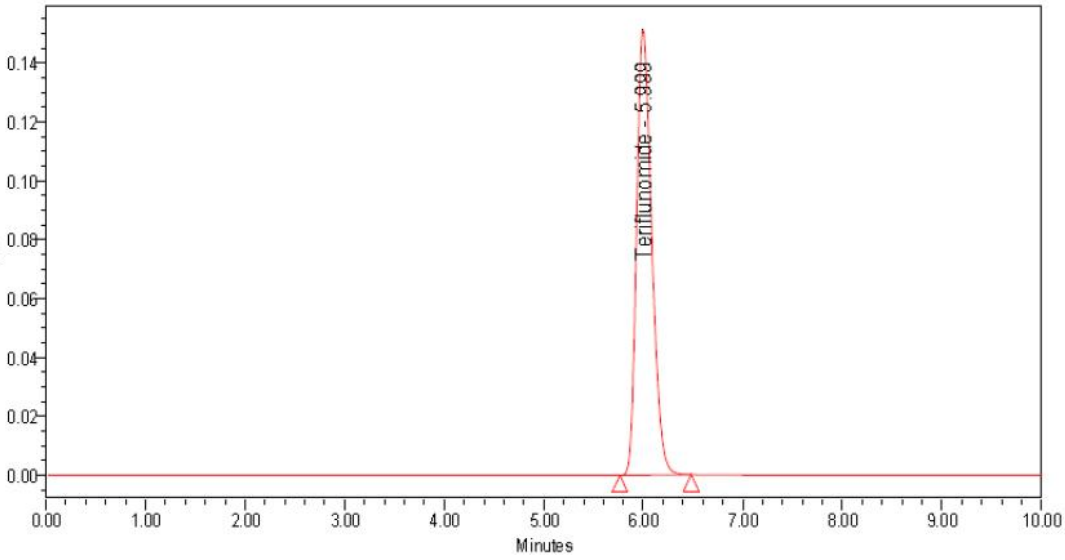
0.45µ PVDF filtrate sample, 5 mL discarded
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	13	1	5.997	1582636	7329		1.27	BB

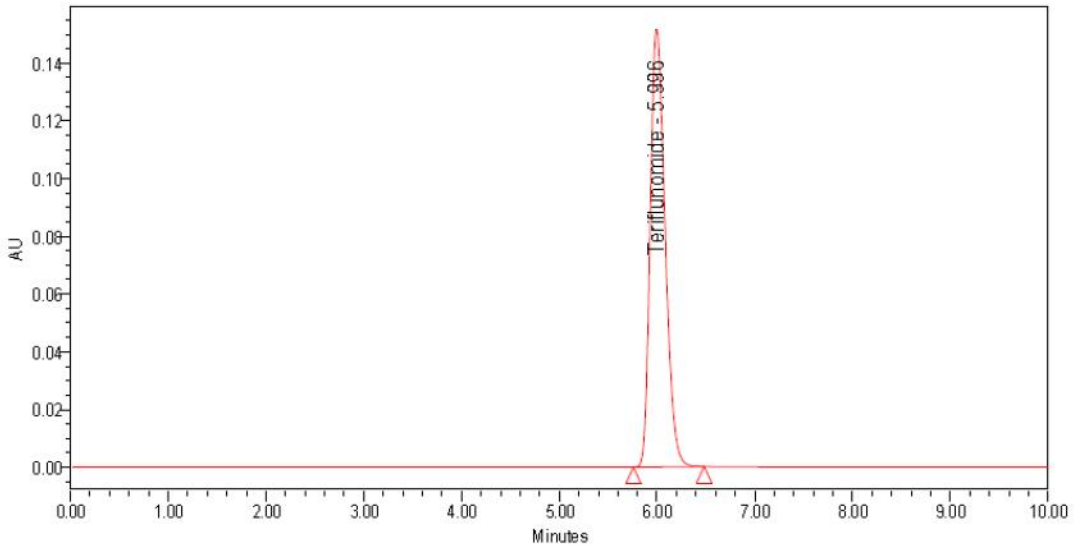
Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	13	2	5.999	1596210	7308		1.29	BB

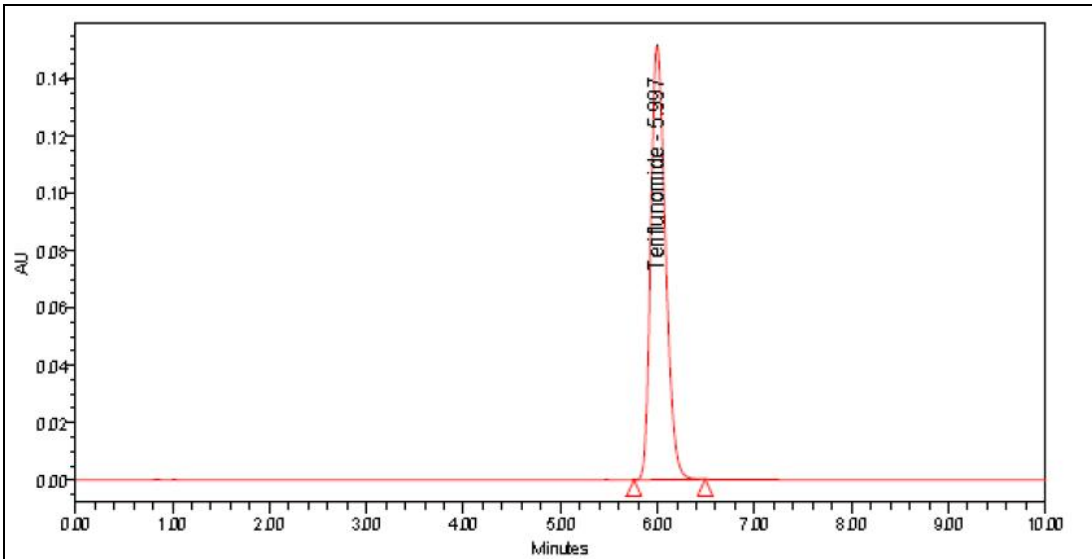
0.45µ PVDF filtrate sample, 6 mL discarded
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	14	1	5.996	1595833	7329		1.28	BB

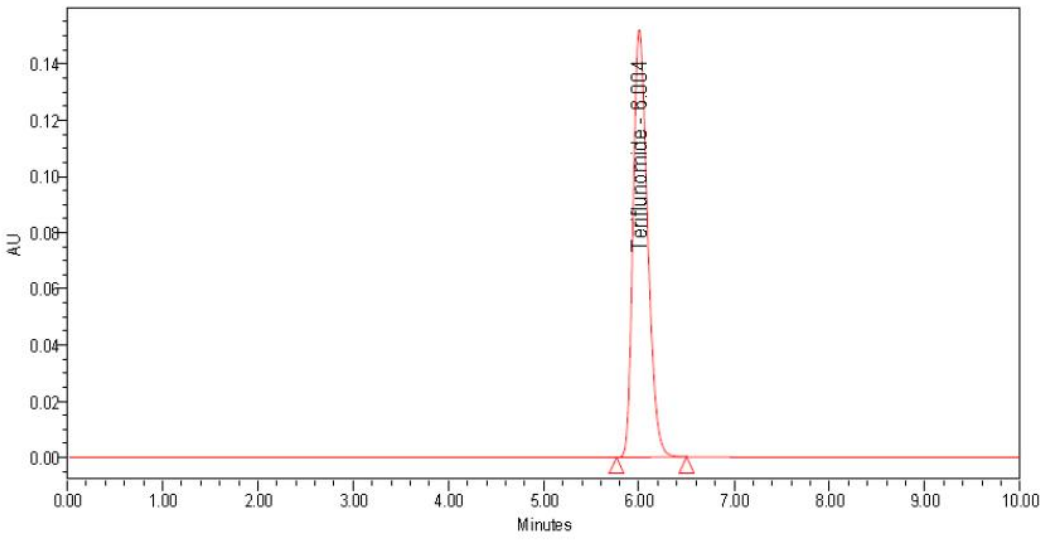
Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	14	2	5.997	1593952	7331		1.28	BB

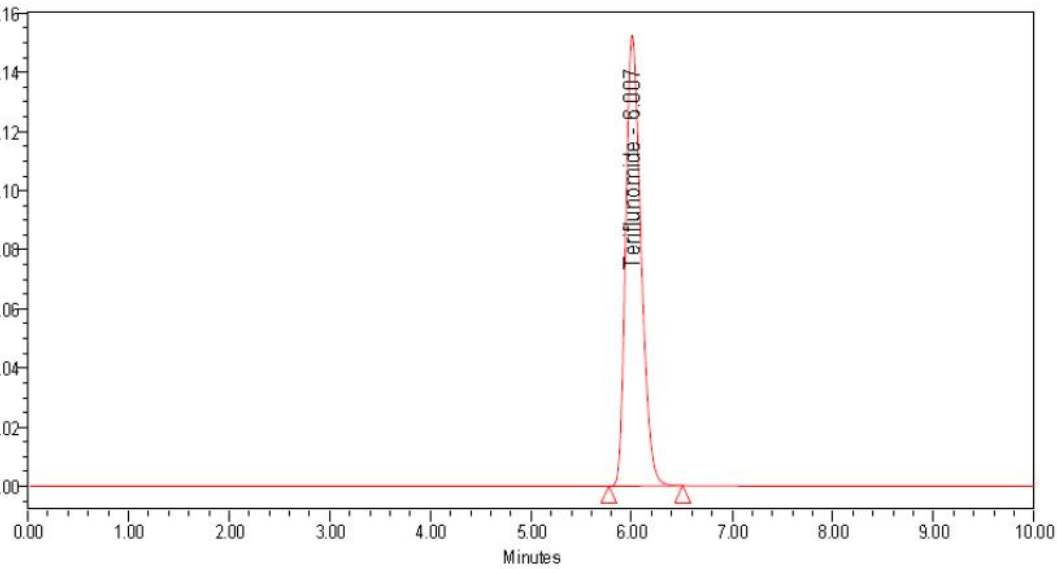
0.45µ Nylon filtrate sample, 3 mL discarded
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	15	1	6.004	1609255	7271		1.29	BB

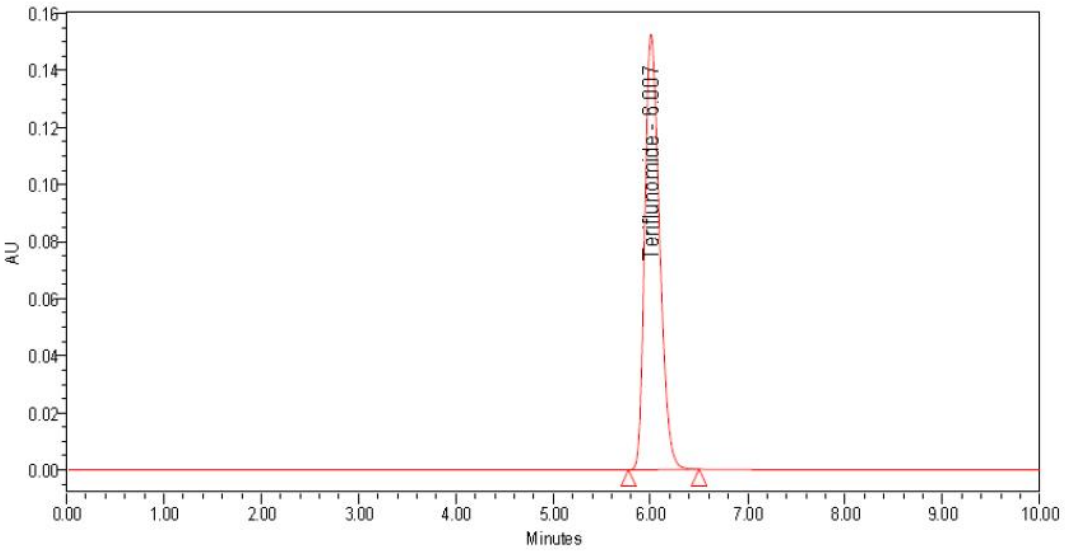
Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	15	2	6.007	1608293	7315		1.30	BB

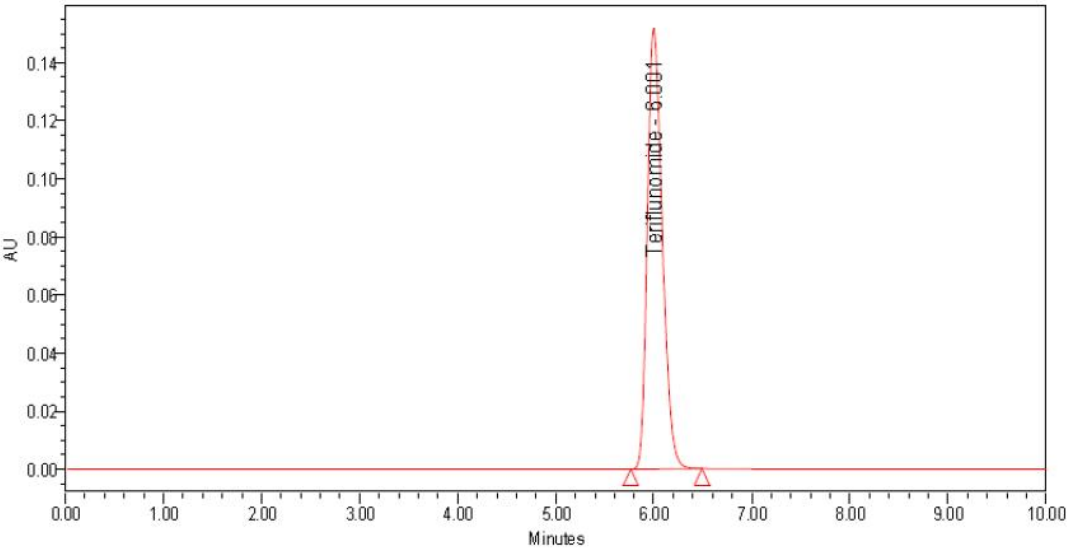
0.45µ Nylon filtrate sample, 4 mL discarded
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	16	1	6.007	1608853	7317		1.29	BB

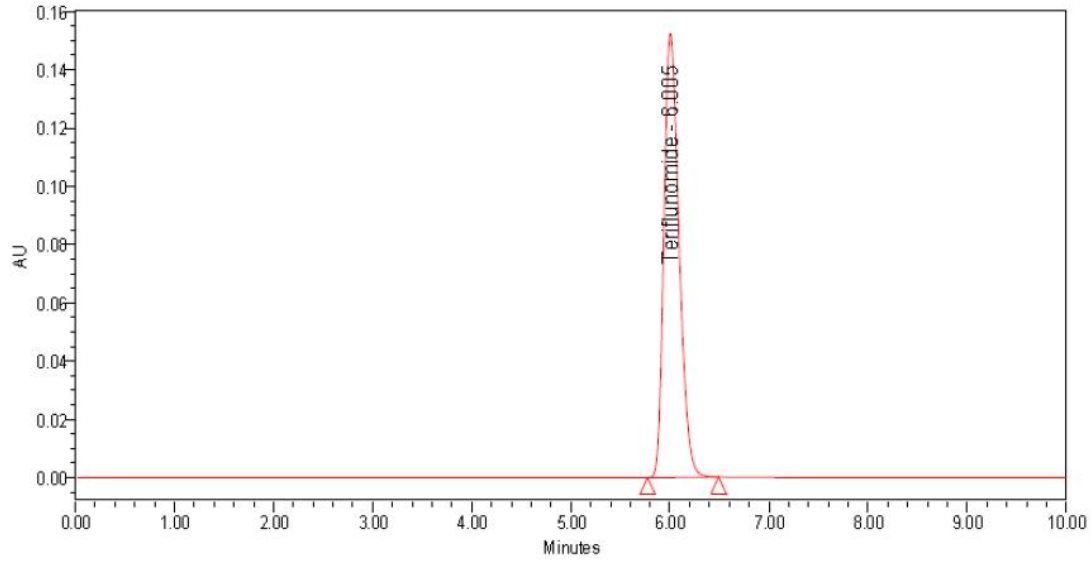
Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	16	2	6.001	1603049	7355		1.28	BB

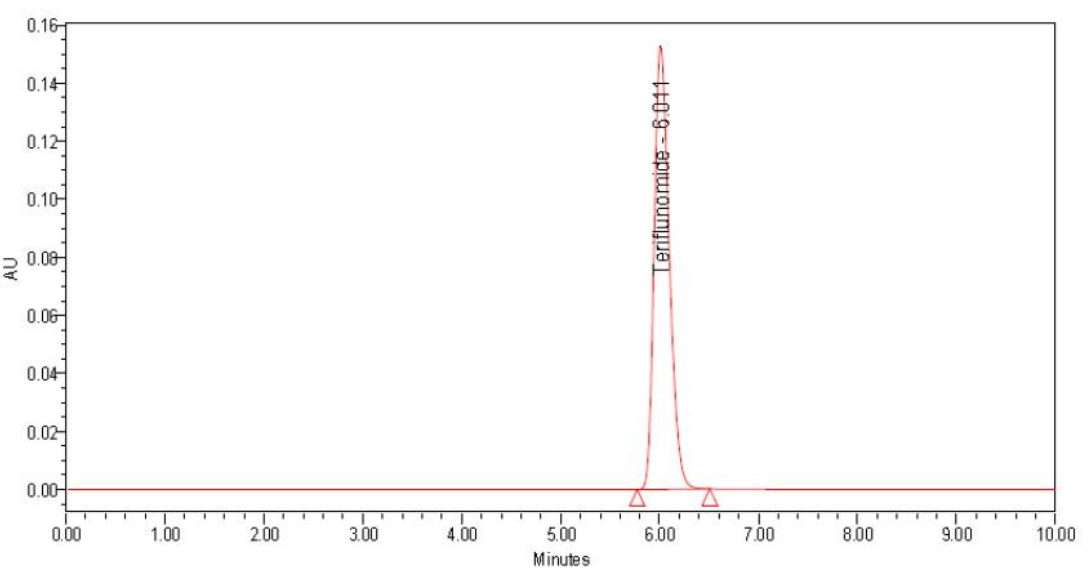
0.45µ Nylon filtrate sample, 5 mL discarded
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	17	1	6.005	1608498	7298		1.29	BB

Injection-2

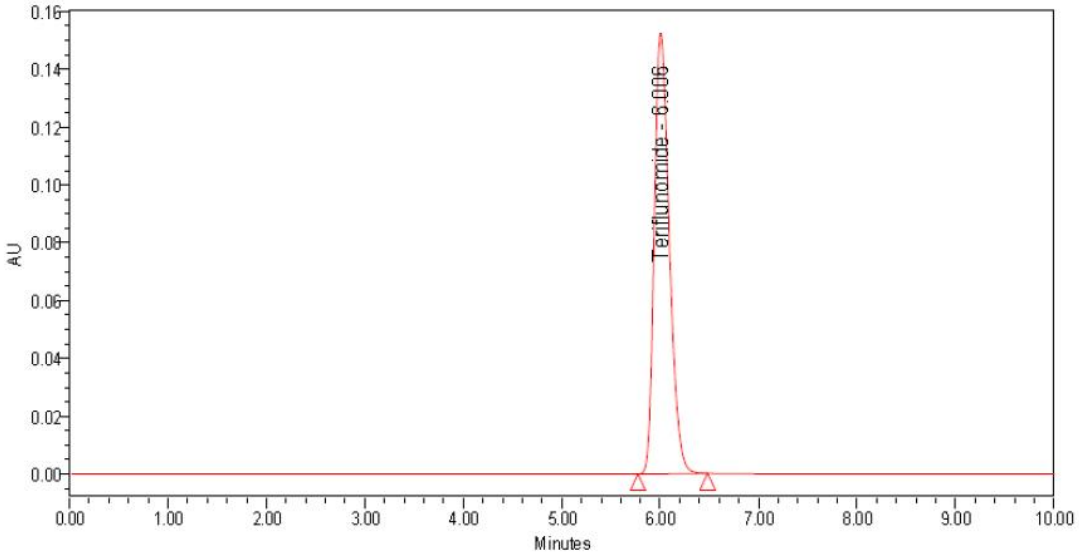


Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	17	2	6.011	1617698	7261		1.29	BB

0.45µ Nylon filtrate sample, 6 mL discarded

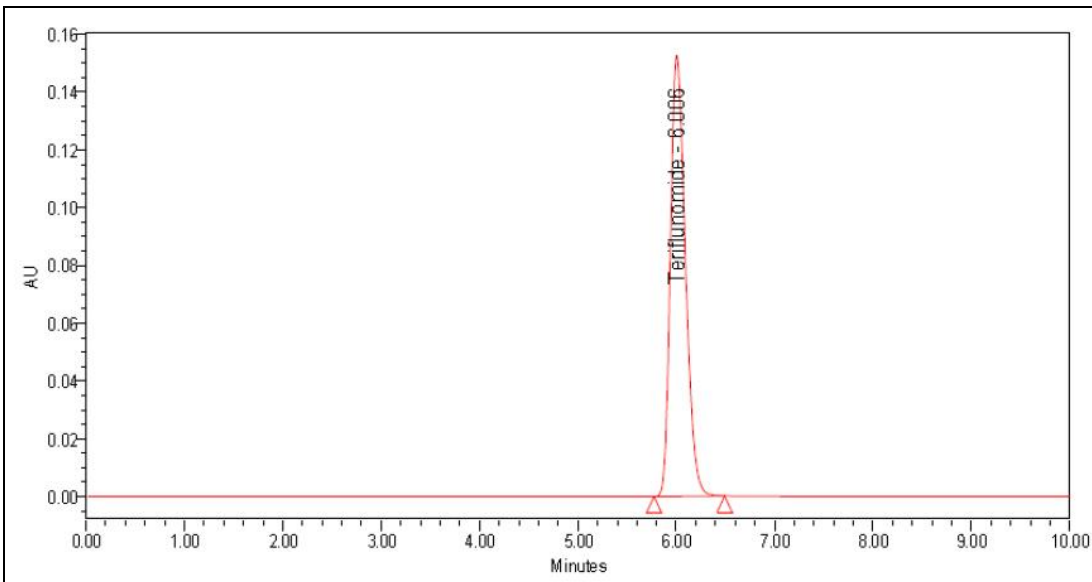
Injection-1



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	18	1	6.006	1612765	7298		1.30	BB

Injection-2



Peak Results

	Name	Vial	Injection	RT	Area	USP Plate Count	USP Resolution	USP Tailing	Int Type
1	Teriflunomide	18	2	6.006	1609565	7300		1.29	BB

8.0 SUMMARY

A simple Reverse Phase High Performance Liquid Chromatographic method has been developed and subsequently validated for Teriflunomide tablets.

The separation was carried out by using a Buffer : acetonitrile (65:35). The detection was carried out at 250nm. The column was Zorbax Eclipse XDB, C8,150 x 4.6mm, 5µl. The flow rate was selected as 1.5ml/min.

The Retention time of Teriflunomide tablets was found to be 6.0. The asymmetry factor or tailing factor of Teriflunomide tablets was found to be 1.2, which indicates symmetrical nature of the peak. The number of theoretical plates of Teriflunomide tablets was found to be 7391, which indicates the efficient performance of the column. These parameters represent the specificity of the method.

From the linearity studies, specified concentration levels were determined. It was observed that Teriflunomide tablets were linear in the range of 5% to 150% for the target concentration by RP-HPLC. The linearity range of Teriflunomide tablets 5% to 150% was found to obey linearity with a correlation coefficient of 0.999.

The validation of the proposed method was verified by system precision and method precision by RP-HPLC. The %RSD of system suitability for Teriflunomide tablets was found to be 0.25.

The validation of the proposed method was verified by recovery studies. The percentage recovery range was found to be satisfied which represent in results. The robustness studies were performed by changing the flow rate, filters and wavelength. The ruggedness study was also performed.

The analytical method validation was carried out by RP-HPLC as per ICH guidelines and given below are the tables are the summary of the results.

Analytical method validation report for Teriflunomide tablets

Table No. 27

S. NO.	TEST	ACCEPTANCE CRITERIA	RESULTS
1.	System Suitability	The USP Tailing factor of Teriflunomide peak is NMT 2.0 from standard preparation. The USP Plate count for Teriflunomide peak NLT 2000 from standard preparation. The RSD of Teriflunomide peak area is NMT 2.0% from five replicate injections of standard preparation.	USP Tailing: 1.27 USP plate count: 7147 RSD: 0.25%
2.	Accuracy	The recovery at each level must be 97.0% to 103.0%. The RSD of all determinations at each level should be not more than 2.0%.	Average mean Recovery at 5% Level: 99.63% RSD: 0.45 % Average mean Recovery at 50% Level: 100.44% RSD: 0.21 % Average mean Recovery at 100% Level: 101.05% RSD: 0.28 % Average mean Recovery at 200% Level: 101.15% RSD: 0.73 %

S. NO.	TEST	ACCEPTANCE CRITERIA	RESULTS
3.	Method Precision	All assay values should be within the 90.0–110.0 % of label claim. The RSD of six (6) assay values should be not more than 2.0%.	Assay Mean: 99.13% RSD: 0.99%
4.	Linearity and range	The correlation coefficient square (r^2) must be NLT 0.997	$r^2 = 0.9999$
5.	Selectivity/ Specificity	Any secondary peak arising from forced degradation study should not interfere with the Teriflunomide peak. The peak purity analysis using a photodiode array detector should demonstrate peak homogeneity. No interference should be observed from diluent, all known impurities at the retention time of Teriflunomide. Teriflunomide peak should be separated from the known and unknown impurities peak (USP resolution NLT 1.5).	No interference observed. Demonstrated Teriflunomide peak homogeneity. No Interference observed. Teriflunomide peak well separated from the known and unknown impurities peak USP resolution is more than 1.5

S. NO.	TEST	ACCEPTANCE CRITERIA	RESULTS
6.	Ruggedness	The system suitability acceptance criteria as described in the method must be met.	Analyst 1 USP Tailing: 1.27 USP plate count: 7147 RSD: 0.25% Analyst 2 USP Tailing: 1.39 USP plate count: 7861 RSD: 0.27%
		The % RSD of Teriflunomide from the six sample preparations should be not more than 2.0%.	Analyst 1 % RSD: 0.98% Analyst 2 % RSD: 0.40%
		All assay values should be within the 90.0 – 110.0 % of label claim.	Analyst 1 Assay Mean: 98.44% Analyst 2 Assay Mean: 98.40%
7.	Solution stability	The concentration and % assay difference between the initial and time point for standard and sample solution should be NMT 2.0% from the initial value respectively. Include an appropriate cautionary statement in the method based on the solution stability.	Standard and samples solutions are stable up to 48 hours when stored at 5°C.
8.	Filter Study	Compare the results of the filtered samples with that of the centrifuged sample preparation. Difference between peak area response of centrifuged sample and filtered sample should not be more than 2.0%.	Results of samples filtered by various filters (0.45µm PVDF and Nylon) are comparable with that of the centrifuged sample
9.	Robustness	All the system suitability requirements must be met.	Method is unaffected by deliberate variations in flow rate, column temperature, pH Variation and mobile phase composition.

9.0 CONCLUSION

A HPLC method for Teriflunomide tablets was developed and validated in tablet dosage form as per ICH guide lines. The results of this validation are as summarized in the report. The results are found to be complying with the acceptance criteria for each of the parameter.

Waters Alliance HPLC (Empower software with PDA detector) with Zorbax Eclipse XDB, C8,150 x 4.6mm, 5µ column, Injection volume of 10 µl is injected and eluted with the Mobile phase (Buffer and ACN, in the ratio of 65:35) which was pumped at a flow rate of 1.5 ml at 250 nm. The peak of Teriflunomide was found well separated at 6.0 min. The developed method was validated for various parameters as per ICH guidelines like system suitability, accuracy, precision, linearity, specificity, ruggedness, robustness and solution stability.

Hence it is concluded that the assay method is found to be valid in terms of reliability, precision, accuracy and specificity and hence it is suitable for routine analysis as well as for stability analysis.

10.0 BIBLIOGRAPHY

1. Asian J. Research Chem. 2(3): volume 02, Issue 03, July ,September,2009
2. Chinese Journal of Analysis Laboratory;2007-10 WU Ji1,WANG Hua-dong2 LIU Hong-wei1,QU Ling-bo2(1.The Commercial Scientific Research Limit Companies of Henan; Province,Zhengzhou,China 2.Department of Chemistry,Zhengzhou University,Zhengzhou 450052,China)
3. Chromatographia Volume 67,Numbers 1-2, Sudhir S. Kamat, Vishal B. Choudhari
4. Ewing G. W., *Instrumental Methods of Chemical Analysis,* McGraw Hill Publishing Company Ins., 2nd Ed., 1960: 3.
5. Hohat H. Willard., Lunne L. Merrit, John A. Dean. Instrumental methods of analysis, 7th edition New Delhi: CBS Publishers; 1999
6. Instrumental methods of analysis, 7th Edn., CBS Publishers, New Delhi.
7. *International Conference on Harmonization*, Validation of Analytical Procedures: Methodology, Federal Register, Nov. 1996:1-8.
8. *International Conference on Harmonization*, Draft Guideline on Validation of Analytical Procedures, Definitions and Terminology, Federal Register (26), 1995: 11260.
9. J AOAC Int. 2010 Jul-Aug;93(4):1207-14. Ganpat University, S.K. Patel College of Pharmaceutical Education and Research, Department of Pharmaceutical Chemistry.
10. J Chromatogr B Analyt Technol Biomed Life Sci.2007 Jul 1;854 Laboratory of Clinical Psychopharmacology, Beijing Anding Hospital, Capital Medical University, China. Guo W, Li W, Guo G, Zhang J, Zhou B, Zhai Y.

11. Jeffery G. H., Bassett J., Medham J.and Denney R. C., *Vogel's Textbook of Quantitative Chemical Analysis,* English Language Book Society/ Longman, 5th Ed., 1989 :668.

12. Journal of Pharmacy Research, Vol 4, No 6 (2011). Paras N. Raveshiya1 and Hetal R. Prajapati.

13. Journal of Liquid Chromatography & Related Technologies, Volume 33**,** Issue 19**,** 2010 , Dilek Dogrukol-Ak[a] & Duygu Yeniceli[a]

14. Journal of Liquid Chromatography & Related Technologies Volume 31, Issue 5, 2008 , Preview V.Nagaraju&A.M.Crouch.

15. Lloyd R, Snyder, Joseph J, Kirkland, Joseph L, Glajesh. Practical HPLC method development. 2nd edition, 1997, pp 1-14.

16. Lurie S. Ira and Wittwer, D. John Jr., *HPLC in Forensic Chemistry*, vii.

17. Sharma, B.K. Instrumutal Methods of Chemicals Analysis. 13th edition., Goel Publisher House, Meerut, **1994**, 7.

18. Skoog D. A., West D. M. and Holler F. J., *Fundamentals of Analytical Chemistry,* Saunders College Publishing, New York, 6th Ed., 713

19. Synder K.L, Krikland J.J and Glajch J.L: Practical HPLC Method Development 2nd Edn, Wiley-Interscience Publication, USA, 1983, 653-661.

20. Talanta. 2010 Jun 30;82(1): 398-403.Epub 2010 May 7.Bioelectro chemistry Laboratory, Chemical and Pharmaceutical Sciences Faculty, University of Chile. Pérez-Ortiz M, Muñoz C, Zapata-Urzúa C, Alvarez-Lueje A.

21. *United State Pharmacopoeia,* Vol. I & II, Asian edition, United Pharmacopoeial Convention, Inc., Rockville, 2000:1923.

22. *United State Pharmacopoeia,* Vol. I & II, Asian edition, United Pharmacopoeial Convention, Inc., Rockville, 2000: 2149.
23. Vijay Malik, Drugs and Cosmetics Act 1940,16th Edn., Eastern Book Company,Lucknow,5
24. Willard H.H, Merritt L.L, Dean J.A. and settle F.A: Instrumental Methods of analysis,7th Edn, CBS Publishers and Distributors, New Delhi 1988, 436-439.
25. www.applgate.co.uk
26. www.drugbank.ca/drugs/ DB00710
27. www.en.wikipedia.org/wiki/Analytical_chemistry.
28. www.elchem.kaist.ac.kr/vt/chem-ed/sep/lc/hplc.htm
29. www.ijpsonline.com/article.as
30. www.ncbi.nlm.nih.gov/pubmed.
31. www.pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=7847419
32. www.rxlist.com/strattera-drug-patient.htm
33. www.scribd.com/doc/29394798/Strattera-atomoxetine-product-Monograph - for-Canada
34. www.scribd.com/doc/50027780/atomoxetine-research-papers
35. www.springerlink.com/content/v2up3wv62172176w/
36. www.tandfonline.com/doi/abs
37. www.waters.com